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# Infected cell types in the ovine lung following exposure to bovine respiratory syncytial virus

James Toby Meehan  
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**Infected cell types in the ovine lung following exposure to bovine  
respiratory syncytial virus**

**Meehan, James Toby, Ph.D.**

**Iowa State University, 1991**

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Infected cell types in the ovine lung following exposure  
to bovine respiratory syncytial virus

by

James Toby Meehan

A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of the  
Requirements for the Degree of  
DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

Signature was redacted for privacy.

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For the Major Department

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For the Graduate College

Iowa State University  
Ames, Iowa

1991

If we could first know where we are and whither we are  
tending, then we could better judge what to do and how to  
do it.

Abraham Lincoln

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## GENERAL INTRODUCTION

Infection with respiratory syncytial virus (RSV) is common in man and ruminants.<sup>6,28,54</sup> Antigenically RSV isolates from humans and animal species are heterologous when examined by neutralization kinetics, but all are closely related and are regarded as a single serotype. Subtypes of human isolates are identified using kinetic serum neutralization tests, monoclonal antibodies in immunofluorescent and radio immunoprecipitation assays.<sup>1,41</sup> For the purposes of this thesis , in the literature review no distinction is made between isolates.

Bovine respiratory syncytial virus (BRSV) is common in co-infections with other respiratory pathogens such as parainfluenzavirus 3 and Pasteurella hemolytica but BRSV is often the sole agent associated with acute fatal bronchointerstitial pneumonia in weanling cattle.<sup>6,12,28</sup> The incidence of disease associated with BRSV infection in cattle is unknown but is much less than the incidence of BRSV infection, which is 60-80%.<sup>6</sup>

Two hallmarks of RSV infections are recurrence and marked variability in disease associated with infection. Atypical interstitial pneumonia can result from BRSV infections in weanling cattle. Atypical interstitial pneumonia is characterized

histologically by severe necrotizing bronchiolitis, interstitial pneumonitis with Type II pneumocyte hyperplasia, hyaline membranes and emphysema.<sup>6,29</sup> Experimental infection of neonatal calves and lambs with BRSV produces necrotizing bronchiolitis, interstitial pneumonitis and pneumonia with lymphocytic peribronchiolar and septal infiltrates and exudates of neutrophils, lymphocytes and macrophages.<sup>15,17,43,87</sup> The inability to produce atypical interstitial pneumonia by experimental infection with BRSV could result from: attenuation of BRSV during laboratory isolation,<sup>33</sup> multiple subtypes of BRSV which vary in virulence,<sup>1,28,41</sup> viral co-infections in animals with atypical interstitial pneumonia,<sup>12,72</sup> and immunopathological reactions caused by natural infections with BRSV that have not been duplicated experimentally.<sup>6,45,46,47,68,92</sup> Evidence supporting any one of these proposals is either lacking or inconclusive.

The composition of respiratory secretions and epithelial lining varies with location within the respiratory tract.<sup>11,29</sup> These variations are responsible for disparity within the respiratory tract regarding susceptibility to insults. Most of the staining for BRSV in infected cattle is within bronchial epithelium.<sup>15,17</sup> Staining for BRSV antigen in experimentally infected sheep is located primarily in bronchiolar and alveolar epithelium.<sup>25,89</sup> The establishment of infection is a critical event in any infectious disease. In reports of cattle and sheep experimentally infected with BRSV, the earliest time period post-inoculation (P.I.) for which lesions and antigen location are described is 2 days.<sup>15,17,25,43,87,89</sup> Several viral replication

cycles can occur in a 2 day time period and secondary foci of infection may be established.<sup>65</sup>

The sites of initial infection within the lung can be determined if tissues are examined at a time interval P.I. that is too brief to allow replication and dissemination of virus. Respiratory syncytial virus replicates in approximately 12 hours in cell culture.<sup>65</sup> This dictates that to determine the site of initial infection, tissues must be examined for antigen between 12-24 hours P.I. Antigen detected after 24 hours P.I. could represent secondary foci of infection. The goals of this study were to i.) determine cell types in the ovine lung that were the first to stain for BRSV antigen, both in infected sheep and in sheep lung slices inoculated in vitro, ii.) examine the chronological progression of infection and development of lesions.

To obtain these goals, the project consisted of the following two steps; first, develop immunocytochemical techniques for detecting BRSV antigen in fixed-processed tissues at the light and electron microscopic levels, second, apply these techniques to lung tissues from infected sheep and to sheep lung tissues that were infected in vitro.

Sheep were chosen as the experimental animal because BRSV causes upper respiratory disease in sheep<sup>31</sup> and lung lesions are produced by experimentally infecting sheep with BRSV.<sup>25,89</sup> We hope to develop BRSV infection in sheep as an experimental model for BRSV infection in cattle and humans. Sheep are more easily handled than cattle and less expensive. In addition, sheep are susceptible to infection with many

viral agents that are pathogenic to cattle. Susceptibility to the same viral pathogens will allow examination of the role of viral co-infections in ruminant respiratory disease.

The sheep were infected by flooding the right cranial lobe of the lung with BRSV. This method of inoculation was chosen to insure exposure of sampled lung tissue to virus and to infect high numbers of cells. To determine whether BRSV has a tropism for a particular epithelial cell type widespread infection is necessary to allow morphometric analysis. Two important differences between our study and previous studies are: detection of BRSV antigen at a time interval of less than 2 days P.I., and use of an immunoperoxidase technique for detection of BRSV antigen in fixed tissues. The immunoperoxidase technique is more sensitive than the immunofluorescent technique<sup>42,50</sup> used by most previous authors.<sup>15,17,87,89</sup>

This dissertation is presented in the alternate format and consists of 2 manuscripts prepared for submission to refereed scientific journals. The format is that of "Veterinary Pathology". A literature review precedes the first manuscript. The first manuscript is published in "Veterinary Pathology", and the second manuscript will be submitted to "Veterinary Pathology". A general summary and discussion follows the last manuscript. A list of references is presented at the end of each manuscript. Literature cited in the introduction, literature review, general summary and discussion is presented at the end of the dissertation.

Members of the National Animal Disease Center - National Veterinary Services Laboratory Animal Care and Use Committee reviewed the protocol for this project and concluded that animals would be humanely treated in this research.

The Ph.D. candidate, James Toby Meehan, was the principal investigator.

## LITERATURE REVIEW

There is much interest in RSV because of the prevalence of infection among humans and ruminants, the disease caused by RSV being particularly severe in neonatal and weanling animals.<sup>5,29,54</sup> Although RSV infection and disease does not naturally occur in laboratory animals such as rats and mice,<sup>54</sup> much of the information regarding RSV infection has been obtained by experimentally infecting these species with RSV. Extensive reviews of RSV infections in humans and ruminants and of the virus recently have been published.<sup>48,54</sup> This review is limited primarily to RSV infection in ruminants. The involvement of RSV infections in establishing secondary bacterial bronchopneumonias will be mentioned briefly.

Respiratory syncytial virus

Respiratory syncytial virus infection is prevalent in humans and ruminants.<sup>33</sup> Infection with RSV causes coryza, bronchiolitis, interstitial pneumonitis and pneumonia.<sup>29,54</sup> Respiratory syncytial virus is a negative sense, single-stranded RNA virus of the genus Pneumonovirus in the paramyxoviridae family.<sup>33</sup> The paramyxoviridae family has many members, including pneumoviruses (RSV, pneumonia virus of mice), paramyxoviruses parainfluenzavirus-3, Sendai virus, New Castle Disease virus, mumps virus), and morbilliviruses (measles virus, canine distemper virus, rinderpest virus).<sup>33</sup>

With the exception of RSV, which lacks a hemagglutinating glycoprotein, members of Paramyxoviridae have characteristic hemagglutination (H or HN) and fusion ( $F_0$ ) glycoproteins associated with their envelopes. Characteristics of these glycoproteins vary among viral genera. For example, the hemagglutinin (HN) of paramyxoviruses has both hemagglutinating and neuraminidase activity, whereas the hemagglutinin glycoprotein (H) of morbilliviruses has no neuraminidase activity.<sup>21</sup>

Respiratory syncytial virus also differs from other members of Paramyxoviridae in its number of genes (10 compared to 6 or 7 for other paramyxoviruses) and its gene order. These differences are evidenced by several proteins in RSV, in particular the G glycoprotein, which are distinct from proteins of other paramyxoviruses.<sup>22,54</sup> Glycoproteins G and  $F_0$  of RSV are envelope-associated. Glycoprotein G is extensively glycosylated, which is unusual for a paramyxovirus, and is responsible for viral attachment to the host cell membrane.<sup>22</sup> The  $F_0$  glycoprotein of RSV differs from fusion proteins of other paramyxoviruses in that its amino terminus is extracellular and the carboxyl terminus is intracellular.<sup>75</sup> The  $F_0$  glycoprotein is cleaved intracellularly by a host cell protease to form the disulfide-linked subunits  $F_1$  and  $F_2$ .<sup>64</sup> The  $F_1$  and  $F_2$  subunits are essential for fusion of viral envelope with the host cell membrane. This fusion results in infection and syncytia formation. The M protein is a structural protein associated with the inner portion of the viral envelope and the N protein is a nucleocapsid protein.<sup>54</sup>



Immune responses are directed against the G, F<sub>0</sub>, N and M proteins of RSV.<sup>59,93</sup> Cell-mediated immunity is directed in part against the N protein.<sup>54,62</sup> Antibodies to the F<sub>0</sub> and G glycoproteins and the M protein are neutralizing<sup>54,59,82</sup> with the F<sub>0</sub> glycoprotein possessing more neutralizing epitopes than the G glycoprotein.

Respiratory syncytial virus is a monotypic strain virus with isolates from all host species (ovine, bovine, caprine, human) cross-reacting on serum neutralization and immunofluorescence tests.<sup>54,63</sup> Using monoclonal antibodies and kinetic serum neutralization tests, human isolates are characterized as subtypes A or B.<sup>1,36,58,81,91</sup> Subtypes are based on differences in the G glycoproteins. The composition of the G glycoprotein also differs in composition between isolates but F<sub>0</sub> is highly conserved and contributes to cross-reaction between subtypes and isolates.<sup>54</sup> Subtypes of bovine RSV have not been described.

As with other paramyxoviruses, infection with RSV is spread to non-infected cells by virions budding from cell membranes and by fusion of infected cells with adjacent noninfected cells. Respiratory syncytial virus virions are highly pleomorphic and bud as either round or filamentous particles (100 nm x 1-4  $\mu$ m) from the apical membranes of infected cells (Figure 1).<sup>4,54</sup> Respiratory syncytial virus is fastidious and highly cell-associated with up to 90% of the virus being cell-associated during an infection.<sup>4,65</sup>

### Epidemiology of respiratory syncytial virus infection

Respiratory syncytial virus was initially isolated from the upper respiratory tract of chimpanzees in 1956.<sup>57</sup> In 1957, the virus was isolated from a human infant with croup.<sup>19</sup> Serological evidence obtained in 1968 demonstrated RSV infection in cattle and the virus was isolated from cattle in 1970.<sup>27,63</sup> Serological evidence for RSV infection in sheep was presented in 1973,<sup>7,26</sup> and isolation of RSV from sheep was reported in 1984.<sup>52</sup> Respiratory syncytial virus has also been isolated from goats and wild bighorn sheep.<sup>77,78,83,86</sup> Serological evidence indicates that horses, swine, dogs, and cats are also susceptible to infection with RSV.<sup>73</sup> Respiratory syncytial virus infection is prevalent in cattle and sheep from many countries, with 60-80% of these species being seropositive.<sup>5,7</sup> However, the incidence of respiratory disease caused by RSV infections in cattle and sheep is not known.<sup>5</sup> In cattle herds infected with RSV, morbidity often approaches 100%.<sup>6,93</sup> Mortality is variable and can be as high as 20%.<sup>5</sup> Disease resulting from RSV infection in cattle is most common among calves in fall and winter seasons.<sup>5</sup> Severe cases of respiratory disease caused by RSV infection is associated with animals on high energy diets.<sup>5,15,28</sup>

### Transmission of respiratory syncytial virus

Respiratory syncytial virus can be transmitted by direct contact, aerosol, and fomites.<sup>38</sup> However, the predominant mode of transmission of RSV among animals is unknown.<sup>38</sup> Respiratory

syncytial virus is very labile and survives only a matter of hours on fomites<sup>38</sup> and in aerosolized droplets.<sup>71</sup> However, these routes of transmission could be significant among closely housed animals. Experimental infection can be initiated by inhalation of virus and by inoculation of conjunctiva with virus.<sup>39</sup>

#### Natural respiratory syncytial virus infection

The hallmarks of RSV infections include recurrence<sup>54,78</sup> and variability of associated disease. In cattle, reinfection with RSV has been observed as early as 3 weeks after the initial infection.<sup>81</sup> The one report of natural RSV infections in sheep describes a mild conjunctivitis in lambs with no clinical signs of pneumonia.<sup>31</sup> Cattle infected with respiratory syncytial virus are often concurrently infected with other pulmonary pathogens such as parainfluenzavirus 3, Pasteurella hemolytica, Pasteurella multocida, Mycoplasma bovis, and Haemophilus somnus.<sup>12,40</sup> However, RSV is commonly the sole agent associated with enzootic pneumonia in young calves<sup>93</sup> and atypical interstitial pneumonia in yearling calves.<sup>5,28</sup> Natural infections with RSV in cattle can be asymptomatic, cause mild upper respiratory tract disease characterized by lacrimal discharge, nasal discharge, coughing, and hyperpnea, or cause severe pneumonia.<sup>5,28</sup> In some cattle herds infected with RSV, two stages of the respiratory disease have been described.<sup>5,28</sup> First, mild respiratory disease, characterized by nasal and lacrimal discharge, occurs and is followed by recovery. Then, within several days, there is a sudden

onset of severe dyspnea leading rapidly to death as a result of atypical interstitial pneumonia.

There are no reports of lesions in sheep caused by natural infection with RSV. Gross lesions caused by natural infections with RSV in cattle include atelectasis and consolidation of the cranioventral portions of the lung, failure of the lungs to collapse when the thoracic cavity is opened, septal and subpleural edema, and interstitial and subpleural emphysema involving the caudodorsal portions of the lung.<sup>6,14,18,28,93</sup> In severe disease, emphysema extends into the mediastinum and into subcutaneous tissues of the back and neck. If there is secondary bacterial infection, fibrinous pleuritis and bronchopneumonia may be present.

No histological lesions have been reported in sheep naturally infected with RSV. Histological lesions caused by natural infection of cattle with RSV are characterized by bronchiolitis with necrosis of the bronchiolar epithelium and accumulation of inflammatory cells and debris within bronchiolar lumens. There is atelectasis and alveoli contain lymphocytes, macrophages, neutrophils and fibrin. There is interstitial emphysema and pneumonitis with accumulation of lymphocytes, plasma cells and macrophages in peribronchiolar regions and alveolar septa.<sup>5,18</sup> Syncytial cells are prominent within bronchioles and alveolar spaces. Bronchiolar epithelium often contains eosinophilic intracytoplasmic inclusion bodies.<sup>5,18</sup> Tracheitis and bronchitis are also present. Atypical interstitial pneumonia caused by natural infection with RSV has in addition to the above changes;

extensive Type II pneumocyte hyperplasia, hyaline membranes, and alveolar edema. When RSV infection in cattle is complicated with secondary bacterial infection, a fibrinopurulent bronchopneumonia is present.<sup>28</sup>

#### Diagnosis of respiratory syncytial virus infection

Immunohistochemistry is the most dependable method for positive identification of RSV in infected tissue.<sup>5,86</sup> Fluorescent antibody staining of frozen lung tissue or nasal exudate is used in most diagnostic laboratories. In outbreaks of RSV infection in cattle, there can be atypical interstitial pneumonia, without detectable antigen by immunohistochemical techniques.<sup>34</sup> Failure to detect viral antigen using immunohistochemical techniques may result from RSV antigen in the tissues being bound by the host antibody.<sup>5,34</sup> Indeed, RSV is rarely isolated from lung tissue of naturally infected cattle.<sup>5,34</sup> This infrequent virus isolation may result from both neutralizing antibody and the fastidious nature of the virus.

Making a definitive diagnosis of RSV infection based on serology is often difficult.<sup>34,49,52</sup> A diagnostic fourfold difference in anti-RSV titer between the initial and convalescent stages of disease is seldom detected because clinical signs are often not noticed until several days following infection. In young calves, maternal antibodies against RSV suppress immune responses to the virus.<sup>49</sup>

Experimental respiratory syncytial virus infection

Respiratory syncytial virus infections have been produced experimentally in calves, lambs, cotton rats, ferrets, mice, and non-human primates.<sup>5,15,16,25,69,70,81,83,86</sup> Cross-infectivity among species and resultant microscopic lesions can be demonstrated.<sup>25,43,69,70,83</sup> As in animals naturally infected with RSV, lesions are variable but less severe. The severe lesions seen in yearling calves naturally infected with RSV have not been experimentally reproduced. Lung lesions resulting from experimental RSV infection vary with species, inoculation regimen, and age of the animals. Neonatal animals, experimentally infected with RSV, generally have more extensive and severe lesions than adolescent or adult animals. Laboratory rodents experimentally infected with human RSV have no gross lesions.<sup>37,43,69,70,83</sup> Lambs (1 week-6 months old) infected with RSV develop minimal gross lesions consisting of foci throughout the lungs of hemorrhage and consolidation less than 1 cm in diameter.<sup>25,89</sup> There are no reports of yearling cattle experimentally infected with RSV. Neonatal calves experimentally infected with RSV have atelectasis and consolidation of the cranioventral portions of the lung.<sup>15,17,87</sup> Among the many animals experimentally infected with RSV, emphysematous lesions have only been reported in one neonatal colostrum-deprived calf.<sup>15</sup>

Microscopic lesions vary in animals experimentally infected with RSV. Respiratory syncytial virus infection in laboratory rodents causes minimal rhinitis, bronchitis, bronchiolitis and interstitial pneumonitis with peribronchiolar and perivascular infiltrates of lymphocytes and macrophages; syncytia are scarce or absent.<sup>36,69,70,83</sup> Epithelial necrosis and exudate in airways and alveoli are not prominent. In neonatal to 6-month-old lambs infected experimentally with RSV, there is minimal to moderate multifocal necrotizing bronchiolitis and interstitial pneumonitis and pneumonia characterized by bronchiolar epithelial necrosis with peribronchiolar and alveolar septal infiltrates of lymphocytes and macrophages.<sup>25,89</sup> Terminal bronchioles and alveoli contain cell debris and exudate composed of neutrophils, lymphocytes, macrophages and RBCs. There is Type II pneumocyte hyperplasia several days post-infection.<sup>25</sup> The severity of lesions in neonatal calves experimentally infected with RSV varies.<sup>15,17,87</sup> In one report the lesions are moderate with bronchitis and bronchial epithelial necrosis being predominant. There is also rhinitis, tracheitis and bronchiolitis with epithelial necrosis and accumulations of neutrophils, macrophages and mononuclear inflammatory cells within bronchioles and extending into alveoli adjacent to affected bronchioles.<sup>17</sup> Similar but more extensive lesions are described in another report of cattle experimentally infected with RSV in which there is bronchitis, bronchiolitis and pneumonia with bronchiolar epithelial necrosis and extensive areas of pneumonia. Airway and alveolar exudate consisted of neutrophils and

mononuclear inflammatory cells. Several days post-inoculation there is pronounced bronchiolar and alveolar epithelial hyperplasia.<sup>15</sup> Unlike lesions in sheep,<sup>25,89</sup> experimental infection of calves with RSV produces prominent syncytia within bronchiolar epithelium and alveolar spaces.<sup>15,17,87</sup> There is evidence that syncytia result from fusion of both epithelial cells<sup>17</sup> and alveolar macrophages.<sup>53</sup> Also present in experimentally infected neonatal calves are intracytoplasmic inclusion bodies within epithelial and syncytial cells.<sup>15,17,87</sup> One proposed explanation for the variation in lesions produced by experimental infection in calves is differences in virulence of the RSV isolates used for inoculation.<sup>17</sup>

Location of RSV antigen in experimental infection varies between calves and lambs in a manner similar to the lesion distribution in these species. Respiratory syncytial virus antigen in experimentally infected weanling and neonatal lambs is located primarily in bronchiolar epithelium and as determined by immunofluorescent techniques.<sup>25,88</sup> Virions are seen in association with bronchiolar epithelium and Type I pneumocytes in lambs.<sup>25,89</sup> Neonatal calves experimentally infected with RSV have viral antigen throughout the respiratory tract, although viral antigen is most common within the bronchial epithelium.<sup>15,17,87</sup> Virion buds and nucleocapsid protein are seen within bronchiolar epithelium. Virions and viral proteins are more often associated with ciliated cells than mucous or Clara cells.<sup>16</sup> Neither virions or nucleocapsids have been reported in association with alveolar epithelium of experimentally infected



calves, although RSV antigen is detected in alveolar septa using immunofluorescence techniques.<sup>16</sup>

#### Immunity to respiratory syncytial virus

Protection against infectious agents in the upper respiratory tract of ruminants is provided by locally produced secretory IgA.<sup>51</sup> Protection against infectious agents within the lung is provided primarily by IgG-1 of humoral origin, the primary immunoglobulin in colostrum of ruminants.<sup>51</sup> Passive immunity provides protection against RSV infection in cattle.<sup>34,48</sup> This protection is evidenced by the low incidence of respiratory disease caused by RSV infection in nursing calves. However, natural infection with RSV causes severe respiratory disease in previously vaccinated yearling calves.<sup>45</sup> In one report RSV infection caused severe disease in calves which had anti-RSV IgG-1 in their lungs, but lacked anti-RSV IgA.<sup>46</sup> This report indicates that immunity to RSV infection is inadequate in calves possessing anti-RSV IgG-1, which should be protective in the lung. Indeed, some authors suggest that during RSV infection, immunopathologic mechanisms may contribute to severe atypical interstitial pneumonia.<sup>6,46,47,68</sup>

In general, cell-mediated immunity plays an important protective role in viral infections.<sup>88,93</sup> Cattle infected with RSV or vaccinated with modified live or killed vaccines show RSV-specific lymphoblastogenesis indicative of a possible role of cell mediated immunity in protection against RSV infections. There is no correlation between lymphoblastogenic responses and levels of anti-RSV antibodies indicating that these two responses may be induced by independent

epitopes on RSV.<sup>30</sup> The roles of cell-mediated and humoral responses in immunity to RSV infections in cattle is yet undetermined.

#### Pathogenesis of respiratory syncytial virus infection

Factors that dictate recurrence of infection and severity of respiratory disease in RSV infections in cattle are not resolved. Respiratory syncytial virus infection of a macrophage cell line stimulates the production of IL-1 inhibitor by the infected macrophages.<sup>74</sup> This IL-1 inhibitor could suppress local anamnestic immune responses and leave the respiratory mucosa susceptible to reinfection.<sup>74</sup>

The severe respiratory disease that is produced in cattle naturally infected with RSV cannot be reproduced experimentally. This indicates that additional unknown factors contribute to the severe respiratory disease associated with RSV infection in cattle. Factors to be considered must include; co-infections with other viral pathogens,<sup>12,72</sup> multiple strains of RSV with variations in virulence among strains,<sup>1,41</sup> immunological components,<sup>4,48,68</sup> and variations in pulmonary; structure (epithelial cell types), function (elasticity) and cell metabolism.<sup>76,81</sup>

Co-infections with pulmonary pathogens can have synergistic effects.<sup>2,12,89</sup> The combination of parainfluenzavirus-3 and RSV infections causes severe respiratory disease in cattle.<sup>12,14,32</sup> There is speculation on the role of bovine viral diarrhea virus (BVDV) in severe respiratory disease in cattle naturally infected with

RSV.<sup>72</sup> Co-infections of BVDV/RSV have not been extensively studied and data are not supportive of a synergism (personal communication with Lehmkuhl and Bolin).<sup>12</sup> Combined infections of Mycoplasma bovis and RSV in gnotobiotic calves caused no more severe lesions than when each agent was used independently to infect calves.<sup>85</sup>

Some viral agents become attenuated during laboratory isolation procedures and in vitro propagation.<sup>33</sup> Attenuation may be the reason that experimental infection with RSV does not cause severe disease. However, there is no evidence of altered virulence among RSV isolates

Immunological components may contribute to severe respiratory disease in cattle infected with RSV. Type III hypersensitivity is one proposed mechanism.<sup>6,45,68</sup> It has been postulated that some calves are sensitized to non-neutralizing epitopes, particularly on the F<sub>0</sub> and G viral glycoproteins, by natural infection or vaccination.<sup>6,44,45,60,68,73</sup> The response to subsequent infection would be preferential production of non-neutralizing antibodies, the formation of antigen-antibody complexes, and subsequent initiation of an Arthus reaction resulting in vascular damage.<sup>24</sup> Data from one clinical study of calves (ages 2 weeks to 7 months) with RSV associated respiratory disease found that severe respiratory disease occurred in calves which had high serum levels of anti-RSV IgG-1.<sup>46</sup> Pulmonary arterioles in these calves had minimal intimal swelling and perivascular edema. The authors interpreted these changes as indicative of an Arthus reaction.<sup>24,46,47</sup> Using immunofluorescent techniques, complement component 3 was detected among cells in the

bronchioles and alveoli of these calves.<sup>47</sup> These data are inconclusive with regards to the role of Arthus reactions in RSV infections. The histologic changes characteristic of Arthus reactions are not a prominent component of lesions caused by natural infection of cattle with RSV. Type I hypersensitivity has been postulated to cause severe respiratory disease in RSV infections in humans.<sup>92</sup> This idea is supported by the presence of high IgE levels in nasal secretions of individuals severely affected by RSV infection. Assessment of the role of classes of immunoglobulins in RSV induced disease in cattle is difficult because of the inability to produce severe disease with experimental infection. Data is suggestive of a correlation between secretory IgE levels and disease severity in RSV infected cattle but inconclusive.<sup>80</sup> Levels of IgA and IgG do not correlate with severity of clinical signs in cattle experimentally infected with RSV.<sup>79</sup>

Assessment of the role of inflammatory mediators in severe disease caused by RSV infections is likewise difficult because of the inability to produce severe disease with experimental infections. However in experimentally infected cattle there is a correlation between thromboxane B<sub>2</sub> levels and severity of clinical disease.<sup>35</sup> A trend exists between disease severity and plasma prostaglandin levels but a statistical correlation is lacking.

Anatomical factors may also contribute to the severity of disease caused by RSV infections. Neonates also have increased closing volumes and higher metabolic rates than adults, but the lung surface area to body weight ratio is similar between neonates and adults.<sup>76</sup> These

characteristics decrease the respiratory reserve of a neonate in comparison to an adult. Therefore, neonates are less able than adults to compensate after a respiratory insult. The above mentioned differences between neonate and adult lungs may contribute to the severe respiratory disease caused by RSV in neonatal calves, but fails to explain the severe respiratory disease caused by RSV infection in yearling cattle.

The ability of a pathogenic agent to affect a particular cell population is a major determinant of disease severity and lesion distribution.<sup>5,10,28</sup> For a particular cell population to be infected with RSV, the virus must gain access to the cells, attach, and replicate. For most paramyxoviruses, absorption is not a selective factor in establishing infection.<sup>21</sup> However, the selectivity of the G glycoprotein of RSV, with regard to cellular absorption is not known but could be a selective factor in determining cell types in the lung which can support RSV infection. Cleavage of the F<sub>0</sub> glycoprotein to the F<sub>1</sub> and F<sub>2</sub> subunits by a host cell protease is a critical event in RSV infection because it brings about fusion of the viral envelope and host cell membranes.<sup>20,21</sup> This fusion allows viral nucleic acids and proteins to enter the host cell. Cells lacking the necessary protease(s) for F<sub>0</sub> cleavage are resistant to RSV infection.<sup>54</sup> The respiratory tract epithelium is comprised of a diverse population of specialized cells.<sup>11</sup> In cattle, no specific cell population(s) has been identified as being preferentially infected in animals with severe respiratory tract disease caused by RSV infection.

### Immunohistochemistry

Immunohistochemistry (immunocytochemistry) was first used by Coons et al. in 1942 to detect bacterial antigens.<sup>23</sup> Direct immunohistochemistry consists of labeling a primary antigen-specific antibody with a marker. Indirect immunohistochemistry makes use of a labelled secondary antibody directed against a primary antigen-specific antibody (Figure 2).<sup>9,50,61,67</sup> Indirect techniques greatly increase the versatility of immunohistochemistry because they eliminate the necessity to conjugate a marker to each different antigen-specific primary antibody. Moreover, indirect immunohistochemical techniques increase the sensitivity of antigen detection.<sup>50</sup> The first markers used to label antibodies for use in immunohistochemistry were fluorescent dyes. The autofluorescence of aldehyde groups in fixed tissues limits the use of immunofluorescent techniques primarily to frozen tissue sections. The cellular detail in frozen sections is greatly compromised so that antigen location and detailed histopathological evaluation cannot be assessed in the same tissue section. The use of immunoenzyme and immunogold histochemical techniques allows antigen detection in fixed processed tissues. Cellular detail is maintained in these tissues so that antigen detection and histopathology can be assessed in detail in the same tissue section.

The primary objective of immunohistochemistry is antigen detection with an optimized signal to noise ratio.<sup>42,50,60</sup> Antigen detection

is dependent upon antigen availability in the tissue. An antigen can be unavailable if it is either destroyed or altered during fixation and processing of the tissue. The term "masked" is used to describe antigens which are altered and rendered undetectable, but not destroyed, by fixation and processing of tissues.<sup>50</sup> Masked antigens most likely result from cross-linking of membranes during fixation.<sup>50</sup> If an antigen is destroyed by tissue fixation and processing, frozen sections are often the only suitable samples for immunohistochemistry. As previously mentioned, cellular detail is greatly compromised in frozen sections. Masking of antigens may be overcome by enzymatic digestion or detergent disruption of cross-linked membranes.<sup>50</sup> Respiratory syncytial virus antigens are masked in tissues fixed and processed for light and electron microscopy. Enzymatic digestion has been used in immunohistochemical techniques to unmask RSV antigen for detection at the light microscopic level.<sup>13</sup> Enzymatic digestion causes minimal to moderate loss of cellular detail at the light microscopic level but the ultrastructural detail would be greatly compromised.

Problems of performing immunohistochemistry at the ultrastructural level are compounded by effects of fixation and processing procedures which optimize structural preservation but preclude antigen detection.<sup>3,50</sup> Protocols for maintaining both ultrastructural integrity and antigen availability are numerous. They include: use of fixatives with modified cross-linking abilities, pre-embedding immunohistochemical staining of tissues, etching of epon-embedded thin sections and

resin removal from thin sections prior to labeling. All of these procedures compromise structural integrity and/or definition. The quest to obtain one ideal fixation and processing technique has been fruitless. "The beauty of the fixative will be in the eyes (of antibodies) of the beholder."<sup>50</sup> The investigator must weigh ultrastructural preservation against antigen identification and decide at what point the loss of ultrastructural detail outweighs the benefits of antigen identification. To date, there are no reports of RSV being labeled at the electron microscopic level. Respiratory syncytial virus identification at the electron microscopic level is dependent upon morphology which can be difficult at times because of RSV's pleomorphic nature and resemblance to microvilli.



Figure 1a. Ovine fetal turbinate cells infected with RSV.

Virions (arrow) and viral buds (arrowhead).

Bar = 2  $\mu$ m

Figure 1b. Ovine fetal turbinate cells infected with RSV. Same field as figure 1a higher magnification. Virion (black arrow) and viral buds (black arrowhead) are identified by electron dense layer opposed to inner side of membrane and nucleoproteins (white arrow). Bar = 250 nm.

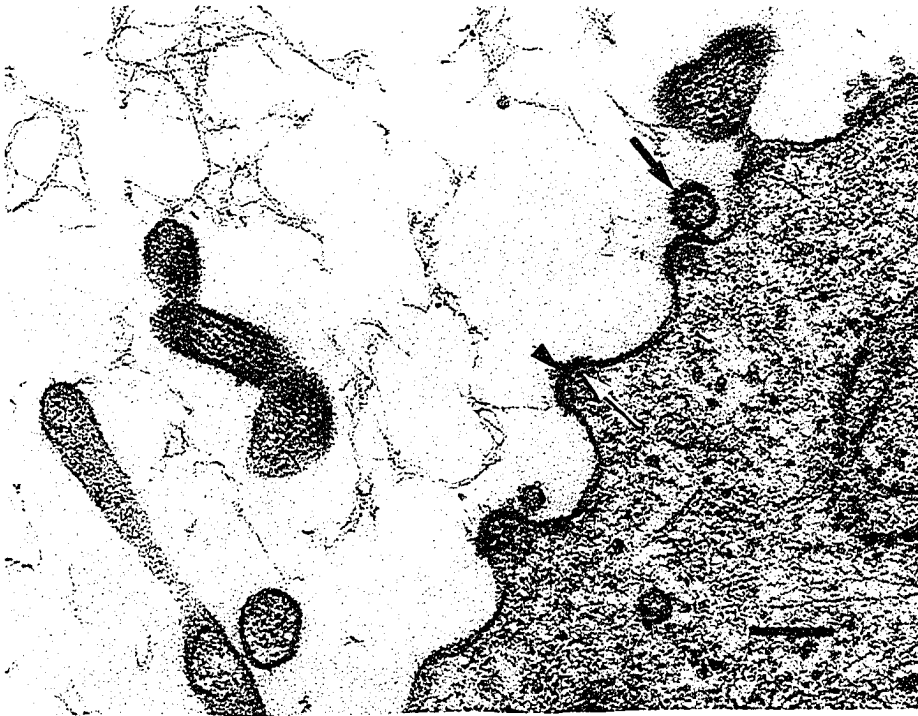
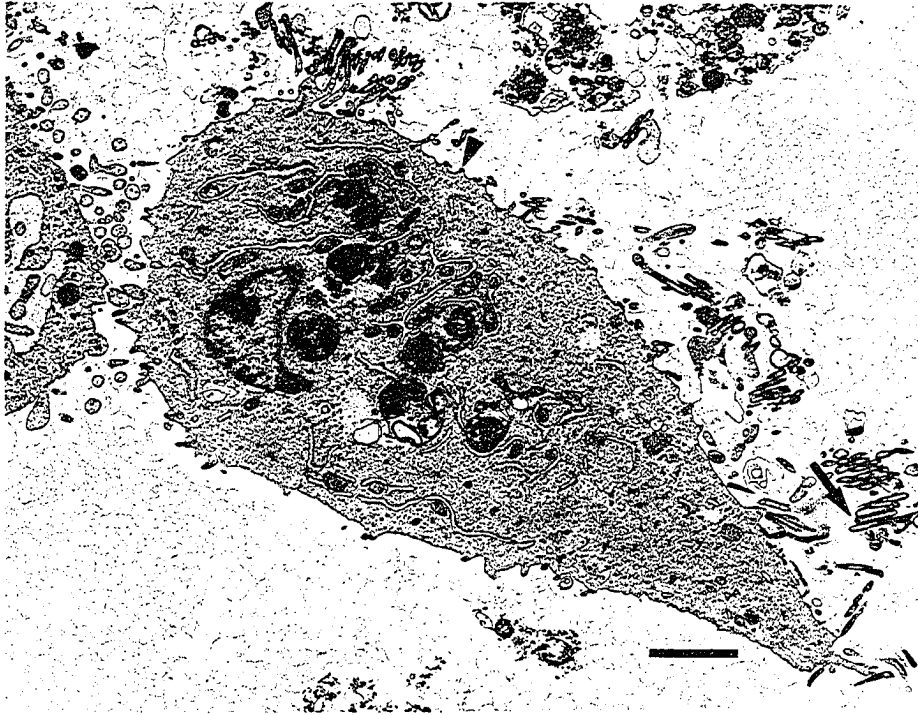
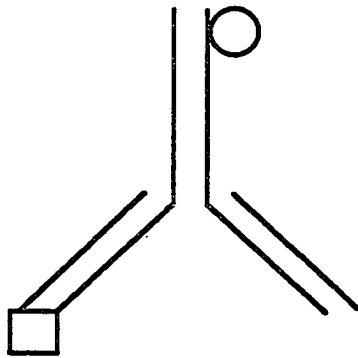
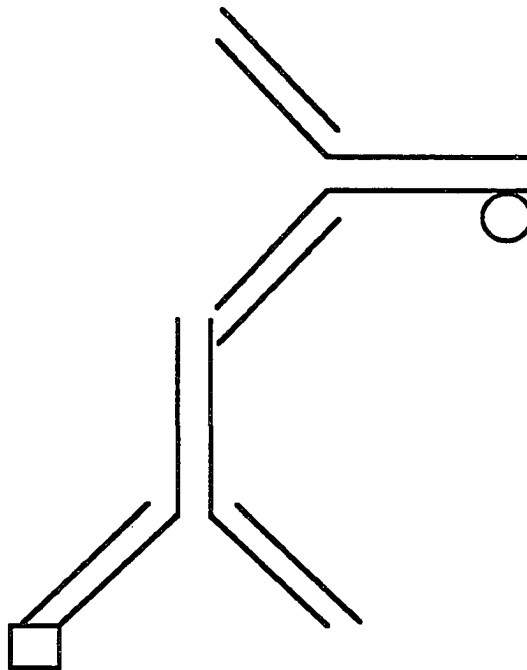


Figure 2. Schematic of direct and indirect immunohistochemical techniques. □ -antigen, ○-marker, ≡ -primary antibody, ≡≡ -secondary antibody



**Direct Immunohistochemistry**



**Indirect Immunohistochemistry**

EVALUATION OF EDTA-TWEEN 20 TREATMENT VS. PROTEASE DIGESTION  
OF FORMALIN FIXED TISSUE SECTIONS FOR DETECTION OF BOVINE  
RESPIRATORY SYNCYTIAL VIRUS ANTIGEN IN INFECTED OVINE LUNG

Evaluation of EDTA-Tween 20 treatment vs protease digestion  
of formalin fixed tissue sections for detection of bovine  
respiratory syncytial virus antigen in infected ovine lung

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## ABSTRACT

The efficacy of protease and EDTA-Tween 20 in unmasking bovine respiratory syncytial virus (BRSV) antigens in formalin fixed lung tissue was compared using avidin-biotin immunoperoxidase procedure. Tissues were from experimentally infected lambs. BRSV antigen stained in both techniques. Treatment with EDTA-Tween 20 resulted in more intense staining of BRSV infected cells, more uniform cytoplasmic staining, less non-specific background and superior cellular detail in comparison to protease digestion.

## INTRODUCTION

Respiratory syncytial virus (RSV) is a common cause of respiratory disease in both humans and livestock.<sup>2,12,14,15</sup> Infection can result in a severe necrotizing bronchiolitis and pneumonia, which are seen more frequently among immature than mature animals.<sup>2,15</sup> Respiratory syncytial virus is a fastidious virus. Histologic lesions allow only a presumptive diagnosis of bovine respiratory syncytial virus (BRSV) pneumonia since parainfluenzavirus 3 can cause similar lesions.<sup>7</sup> Until recently, histological detection of BRSV was done only with fluorescent antibody test on unfixed tissue.

Immunohistochemical techniques using fixed tissue greatly facilitates BRSV diagnosis by allowing identification of specific cell types infected and tissue changes. Recently BRSV antigen detection in fixed tissue has been demonstrated with immunoperoxidase techniques using a monoclonal antibody against BRSV or protease digestion in combination with anti-BRSV rabbit antisera.<sup>4,5,6</sup> Protease tissue digestion, while unmasking some antigenic sites, destroys others and causes noticeable tissue damage.<sup>8</sup> In this study we compare protease digestion with detergent treatment for unmasking BRSV antigen in fixed tissue sections.



## MATERIALS AND METHODS

Yearling sheep were inoculated intratracheally with 20 ml of BRSV 375 inoculum<sup>12</sup> for 2 consecutive days (total 40 ml). The sheep were killed on postinoculation day 5 and lung tissue was fixed by submersion and stored in 10% neutral buffered formalin for 4 days, processed routinely and embedded in paraffin at 60°C. Additional tissue was fixed by submersion in 4% paraformaldehyde in 0.1M cacodylate buffer for 24 hr and in 2.5% glutaraldehyde in 0.1M cacodylate buffer for 12 hr. Tissues were stored in 0.1M cacodylate buffer prior to paraffin embedding. Sections of 3  $\mu$ m thickness were put on poly-L-lysine coated glass slides, deparaffinized with three changes of xylene, rehydrated through graded acidified alcohol baths (0.5 ml concentrated HCl/250 ml) and taken to distilled water. At this point the sections were subjected to one of two treatments prior to staining for BRSV using an immunoperoxidase ABC procedure<sup>9</sup>. One treatment involved protease digestion of the sections, the other was adapted from a technique in which the sections are washed with a buffer containing EDTA and Tween 20<sup>10,13</sup>. The primary antibody in both techniques was rabbit antisera made to the whole virus (Dr. C. Kucera, Norden Lab., Inc., Lincoln, NE) and the remaining immunoperoxidase reagents were obtained commercially (Vectastain ABC Kit, Vector Lab., Burlingame, CA) and used at identical concentrations on all sections.

The technique employing EDTA and Tween 20, was as follows. After rehydration endogenous peroxidase activity was eliminated by

submerging the sections in freshly prepared 1%  $H_2O_2$  in absolute methanol for 30 minutes. The tissues were then rinsed by submersion for three 5 minute periods in 0.05M Tris buffered saline (TBS) pH 7.6 after which they were submersed in TBS-EDTA-Tween buffer pH 7.6 (TBS with 1mM EDTA and 0.05% Tween 20) and incubated for 10 minutes. The sections were then flooded with undiluted normal goat serum (blocking serum) and incubated for 15 minutes at 37°C in a humidified chamber. The blocking serum was blotted off and primary rabbit anti-BRSV antiserum, diluted 1:1000 in TBS, was applied by flooding each section after which they were incubated at 4°C overnight in a humidified chamber. The sections were then rinsed by submersion for three 5 minute periods in TBS-EDTA-Tween buffer. Next the sections were flooded with biotinylated goat anti-rabbit antisera diluted 1:100 in TBS and incubated at 37°C for 60 minutes in a humidified chamber. The sections were then rinsed by submersion for three 5 minute periods in TBS-EDTA-Tween buffer. After rinsing the sections were flooded with ABC reagent, diluted 1:100 in TBS-EDTA-Tween buffer and incubated for 60 minutes at 37°C in a humidified chamber. Following ABC incubation, the sections were rinsed for one 5 minute period by submersion in TBS-EDTA-Tween buffer, followed by two 5 minute rinses with TBS. The sections were then incubated with freshly made DAB substrate for 10 minutes at room temperature in a humidified chamber, followed by a 5 minute rinse with running tap water. All sections were counterstained with Mayers hematoxylin.

The protease digestion technique was as follows.<sup>4,5</sup> After rehydration, endogenous peroxidase activity was eliminated by submerging the sections in freshly prepared 1%  $H_2O_2$  in absolute methanol for 30 minutes. Tissues were then rinsed with tap water for 5 minutes. Next protease digestion was carried out for 10 minutes at 37°C by flooding each section with a solution of 1% protease (Protease XIV, Sigma Chem. Co.) made up in TBS pH 7.6. Tissues were then washed by submersion for three 5-minute periods in TBS. The sections were then flooded with undiluted blocking normal goat serum, as described for the Tris-EDTA-Tween technique. From this point on, the protease and Tween treated sections were treated identically, with the exception that for the protease technique all rinses were carried out and reagents made up using only TBS.

Negative controls consisted of a) substitution of normal rabbit sera for the primary antibody b) substitution of TBS for primary antibody and c.) staining of adenovirus infected sheep lung tissues.

## RESULTS

Histological changes in the infected tissues were characterized by moderate multifocal purulent bronchiolitis with epithelial sloughing, syncytia formation and mild alveolar septa thickening. Specific staining of infected cells was obtained with both methods. In all sections, infected cells consisted of sloughed and intact bronchiolar epithelium, alveolar macrophages and occasionally syncytia.

The two staining techniques were compared by staining paired adjacent sections (Figs. 1a & b through 3a & b). Protease digestion, prior to staining, resulted in peroxidase labeling of infected cells primarily along the cell's periphery and of perinuclear cytoplasmic inclusions, not detectable on routine H & E sections (Figs. 1b & 2b). A diffuse background staining, not extensive enough to interfere with recognition of infected cells, was present throughout the parenchyma and airspaces in the sections exposed to protease digestion. The loss of cellular detail resulting from protease digestion is evident by the lack of cilia on the airway epithelium (Fig. 3b).

Sections that were treated with TBS-EDTA-Tween buffer had intense antigen staining distributed throughout the cytoplasm of infected cells. Cytoplasmic inclusions were not evident (Figs. 1a & 2a). Instead, many of the antigen positive cells contained cytoplasmic vacuoles (Fig. 2a). Cellular detail was preserved with cilia easily visualized (Fig. 3a). In some cells the staining of viral antigens was most intense along the cilia (Fig. 3a), and apical portions of the

cell. There was no diffuse background staining (Figs. 2a & 3a). The TBS-EDTA-Tween technique resulted in more intense and uniform staining of a greater number of cells than the protease technique.

BRSV antigen stained in paraformaldehyde and glutaraldehyde fixed tissues. However, glutaraldehyde fixed tissues stained much less intensely than either formalin or paraformaldehyde fixed tissues.

All negative controls had no peroxidase staining.

## DISCUSSION

In our laboratory the use of TBS-EDTA-Tween buffer technique for detection of BRSV infected cells in fixed processed tissues was superior to protease digestion for the following reasons: 1) intensity and homogeneity of cytoplasmic staining was greater; 2) diffuse background staining was not present; 3) infected cells were more easily distinguished from adjacent non-infected cells; and 4) there were fewer adverse effects on cellular morphology.

Tween 20 acts to unmask antigenic sites in fixed cell membranes through its actions as a non-ionic detergent, as opposed to protease digestion which acts by digesting protein portions of the cell membrane.<sup>8,10</sup> Enzymatic digestion, while exposing some antigenic sites destroys others; therefore, time, temperature and pH must all be carefully controlled to ensure consistent tissue digestion to optimally expose the antigenic sites. Such precise control of these variables is not critical with the TBS-EDTA-Tween technique.

The more intense peroxidase labeling of the TBS-EDTA-Tween treated sections results from Tween's ability to unmask antigens without altering antigenic determinants and lowering surface tension, which allows deeper and more uniform penetration of labelling antibodies and reagents into the section.<sup>10</sup> The TBS-EDTA-Tween treatment of sections decreases non-specific background through better elution of unbound reagents and decreased non-specific binding of antibodies through hydrophobic interactions with the tissue.<sup>10</sup>

The staining characteristics of the protease digested infected tissues are compatible with those described by Bryson et al.<sup>4,5</sup> Inclusions similar to those seen in the protease digested sections were reported with the use of fluorescent antibody techniques and were attributed to differences in antigenicity of BRSV strains and antisera specificity.<sup>1</sup> In contrast, our results indicate the difference in staining is the result of the technique used since our antiserum and viral strains were homologous. Either TBS-EDTA-Tween treatment is not unmasking the antigens comprising the inclusions or staining of inclusions is obscured by the homogenous cytoplasmic staining.

The decreased intensity of staining for BRSV antigen in glutaraldehyde fixed tissues in comparison with either formalin or paraformaldehyde fixation is attributable to the extensive crosslinking and irreversibility of glutaraldehyde fixation.<sup>3,11</sup>

The TBS-EDTA-Tween technique aids in the diagnosis of BRSV by decreasing the non-specific background staining of necrotic and inflamed tissue, prominent in many samples received by diagnostic laboratories. From the research aspect, protease causes loss of cellular detail while the TBS-EDTA-Tween treatment did not cause noticeable tissue damage. This is of major importance for identification and examination of infected cells. For these reasons we feel that the TBS-EDTA-Tween technique is more advantageous than protease digestion for detection of BRSV antigen in fixed tissues for diagnostic and research purposes.

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Figures 1-3.) BRSV infected lung tissue

Figure 1. a.) BRSV infected lung tissue stained with immunoperoxidase

for BRSV antigen after TBS-EDTA-Tween treatment.

Infected cells stain brown. Many contain cytoplasmic vacuoles (arrow). X100

b.) Adjacent section to tissue pictured in Figure 1a stained

with immunoperoxidase for BRSV antigen after protease treatment. Infected cells stain brown, many contain

intracytoplasmic inclusions (arrow). Non-specific

background staining is evident in uninfected cells and

bronchiolar exudate. X100

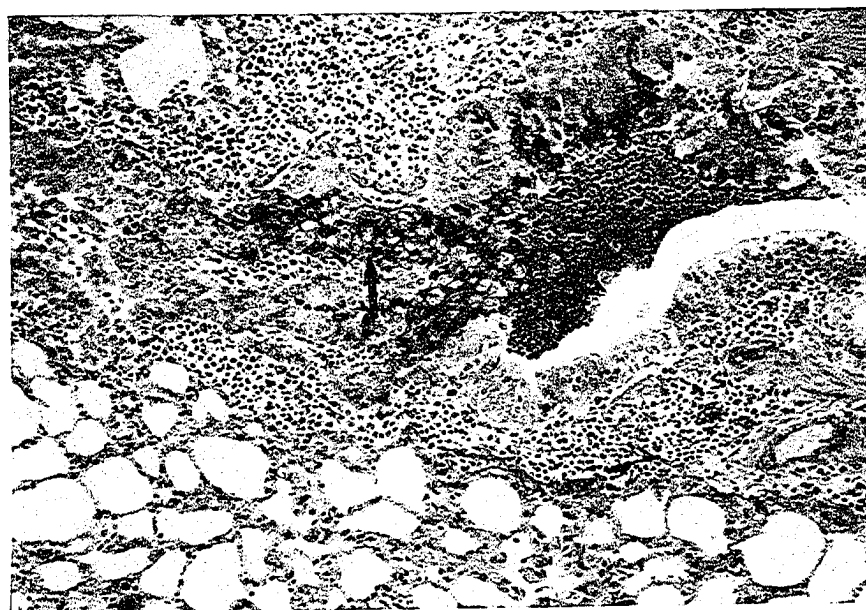
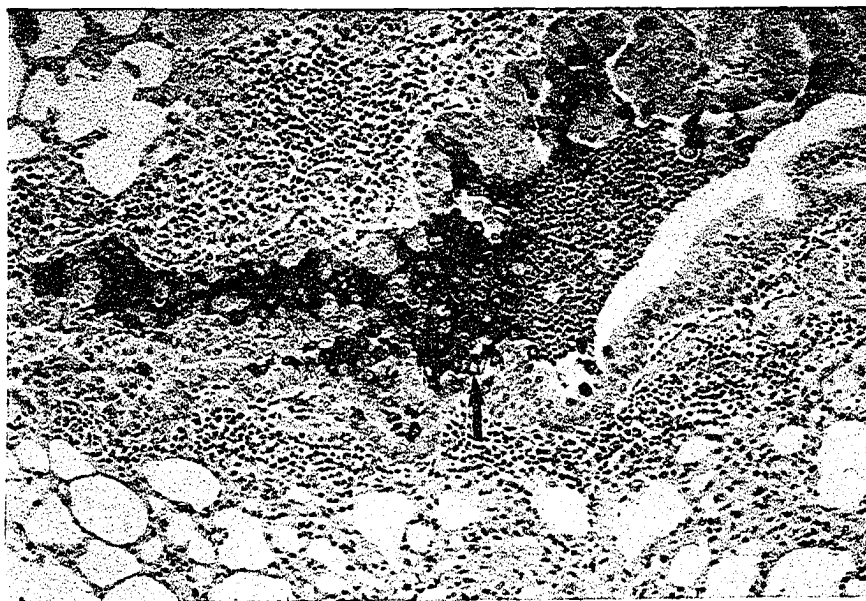


Figure 2. a.) BRSV infected lung tissue stained with immunoperoxidase for BRSV antigen after TBS-EDTA-Tween treatment. Same field as pictured in Figure 1 at a higher magnification. Cellular outlines are distinct and cytoplasm of infected cells stains uniformly, many contain vacuoles (arrow). X250

b.) Adjacent section to tissue pictured in Figure 2a stained with immunoperoxidase for BRSV antigen after protease treatment. Cellular outlines are indistinct and cytoplasmic staining of infected cells is not uniform. Distinct intracytoplasmic inclusions (arrow) are present in many infected cells. X250

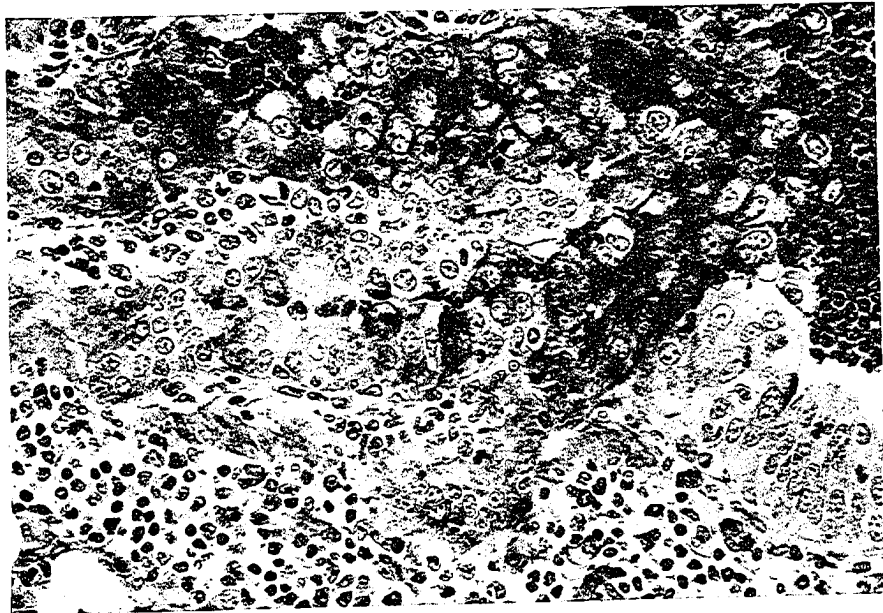
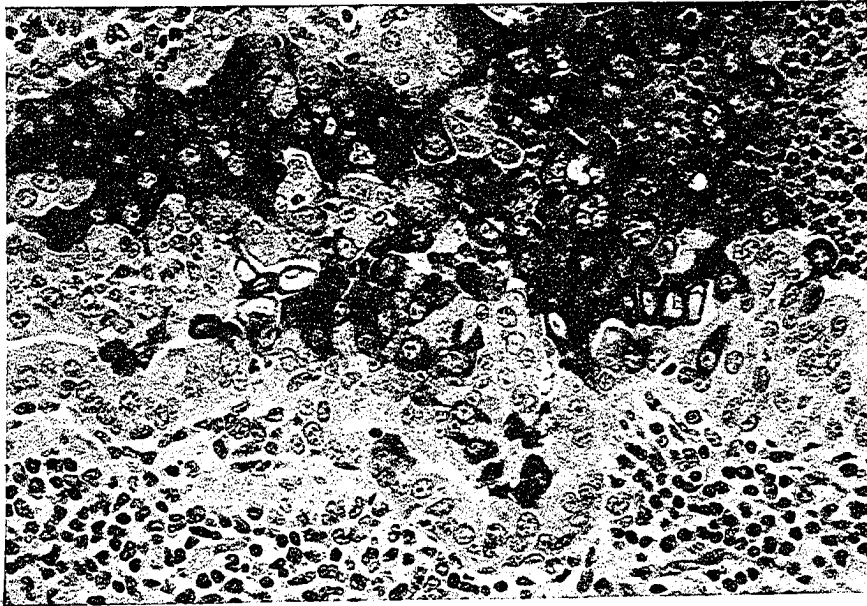
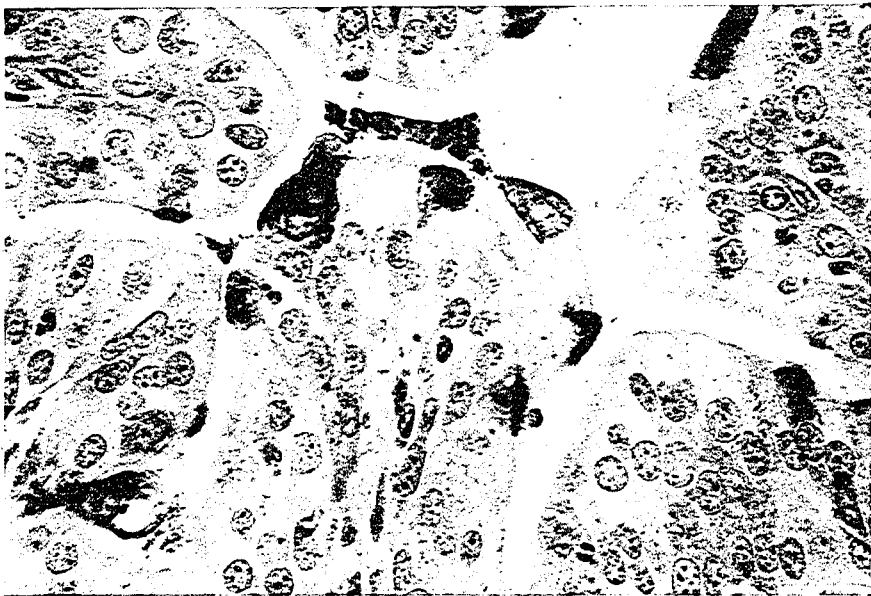
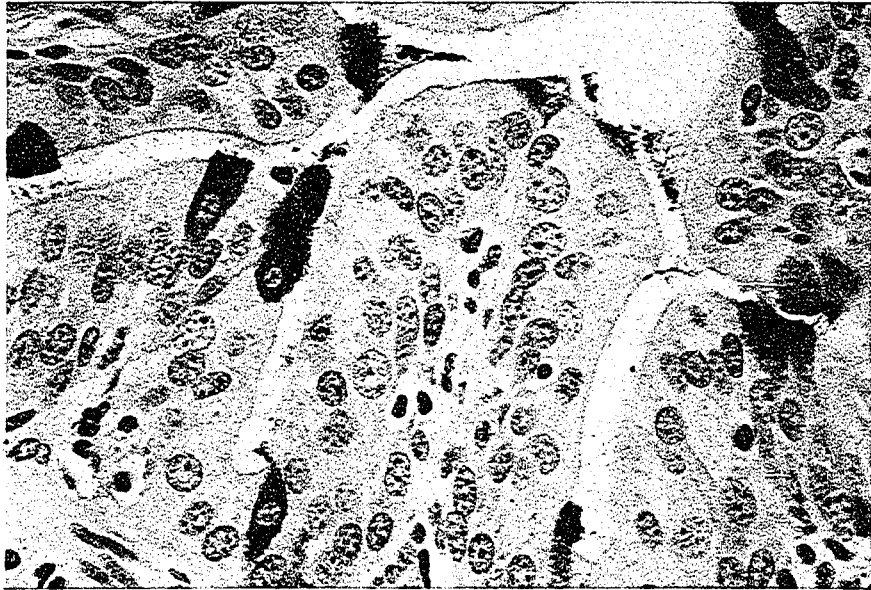


Figure 3. a.) Infected bronchiolar epithelial cells stained for BRSV antigen after TBS-EDTA-Tween treatment. Cilia are evident with staining of viral antigen most intense along cilia and apical cell membrane of some cells. X400

b.) Adjacent section to tissue pictured in Figure 3a stained with immunoperoxidase for BRSV antigen after protease treatment. Cilia are not evident. Loss of other cellular detail and increased non-specific staining is evident. X400





INFECTED CELL TYPES IN OVINE LUNG FOLLOWING EXPOSURE  
TO BOVINE RESPIRATORY SYNCYTIAL VIRUS

Infected cell types in ovine lung following exposure  
to respiratory syncytial virus

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## ABSTRACT

We have determined the first cell types to stain for viral antigen in lung tissue from sheep infected with bovine respiratory syncytial virus (BRSV). Indirect immunoperoxidase, performed on paraffin embedded sections, and transmission electron microscopy were used to determine the location of BRSV antigen at time intervals between 0.5 day and 8 days post-inoculation (P.I.). Respiratory syncytial virus antigen was detected in alveolar macrophages in tissues collected at 0.5 day P.I. Our conclusion is that alveolar macrophages are the first cell type in which BRSV antigen can be detected in the sheep lung following exposure to respiratory syncytial virus but that macrophage infection is rare. Antigen was primarily restricted to bronchiolar and alveolar epithelium. Bronchiolar epithelium and Type I pneumocytes simultaneously stained for BRSV antigen in tissues collected from 1-6 days P.I. and comprised the majority of infected cells. Lung slices inoculated in vitro with respiratory syncytial virus did not stain for BRSV antigen.

## INTRODUCTION

Respiratory syncytial virus (RSV) is a prevalent respiratory pathogen in primates and ruminants.<sup>2,21</sup> It is the major cause of respiratory disease with subsequent hospitalization of children less than 3 years of age.<sup>12</sup> Moreover, bovine RSV (BRSV) is a primary viral respiratory pathogen in bovine.<sup>2,9,35</sup> It is common in co-infections with other respiratory pathogens such as parainfluenzavirus 3 and Pasteurella haemolytica and is often the sole agent associated with acute, fatal interstitial pneumonia and bronchiolitis in yearling cattle.<sup>2,9</sup> The incidence of RSV infection in cattle is 60-80%, but the incidence of disease associated with infection is unknown.<sup>2</sup> An ovine isolate of RSV has been associated with mild respiratory disease in sheep<sup>11</sup> but data regarding incidence of clinical disease is lacking.

Cattle that die from acute respiratory disease caused by BRSV infection commonly develop atypical interstitial pneumonia. The lungs are diffusely edematous, have widespread emphysema and fail to collapse when the thoracic cavity is opened.<sup>3,9</sup>

Histological changes that characterize atypical interstitial pneumonia caused by natural infection with BRSV are severe and diffuse necrotizing bronchiolitis and interstitial pneumonia, with alveolar edema, Type II epithelial cell hyperplasia, hyaline membranes, interstitial emphysema and syncytial cells.<sup>2,9</sup> Eosinophilic, intracytoplasmic, viral inclusion bodies may be present within

bronchiolar epithelium or syncytia. Attempts have failed to experimentally reproduce atypical interstitial pneumonia with BRSV infection in cattle. Indeed, experimental infection with BRSV in neonatal calves and lambs results in bronchitis, bronchiolitis and interstitial pneumonitis with interstitial lymphocytic infiltrates, epithelial necrosis and syncytia, but interstitial emphysema and other changes characteristic of atypical interstitial pneumonia are rare or absent.<sup>6,7,8,31,32</sup> There are no reports of severe lesions in yearling cattle or sheep experimentally infected with BRSV.<sup>33</sup> Considerations for differences between experimental and field cases include; multiple strains of BRSV which vary in virulence,<sup>1,14</sup> attenuation of field strains or selection of avirulent strains during laboratory virus isolation,<sup>12</sup> viral co-infections in field cases,<sup>4,28</sup> and immunopathologic components which contribute to severe respiratory disease in cattle naturally infected with BRSV.<sup>3,15,24,26</sup> Data are lacking or inconclusive for any of the above considerations. For certain, the difference in lesion distribution between natural and experimental infections is indicative of different cell types being affected in the two instances.<sup>8</sup>

A critical event in any infectious disease is the initial site of infection. In calves experimentally infected with BRSV, epithelial necrosis and viral antigen are detected at all levels of the respiratory tract.<sup>6,7</sup> In contrast, in sheep experimentally infected with BRSV, necrosis and antigen are restricted primarily to bronchiolar and alveolar epithelium.<sup>8,32</sup> In experimental studies in cattle and

sheep, BRSV antigen has been detected in lung tissue from animals killed at 2 days post-inoculation (P.I.).<sup>6,7,31,32</sup> Tissues were collected from one calf at 1 day P.I. and examined, but no BRSV antigen was detected.<sup>6</sup> Antigen present in tissues collected at 2 days P.I. is presumably the result of 4 or more viral replications.<sup>25</sup> As a result, the site of initial infection could not be determined because by 2 days P.I. the virus would have spread from the initial infected cell population to adjacent non-infected cells. Moreover, in the majority of field and experimental cases, antigen location has been determined using immunofluorescent techniques.<sup>6,7,31,32</sup> These techniques are less sensitive than immunoperoxidase techniques<sup>17</sup> and greatly compromise histopathological examination of the tissue, so that determination of specific cell types infected is difficult.

Our study had two objectives. First, to determine the initial cell types infected in sheep lung after one exposure to virus. Second, to examine the progression of infection and lesion development. We used an indirect immunoperoxidase technique<sup>22</sup> to detect BRSV antigen in tissues that had been flooded with viral inoculum either in vivo or in vitro<sup>23</sup>. Flooding the lung with viral inoculum assured us that the lung tissue obtained for histological examination had been exposed to virus. We determined BRSV antigen presence at the approximate end of one viral replication cycle. This protocol allowed us to identify the cell types that were first stained for viral antigen after exposure of all levels of the respiratory tract to BRSV. Additionally, we determined antigen location as the infection progressed.

## MATERIALS AND METHODS

In vivo

Two groups of Border Leicester sheep (10-18 mos.) were used. All animals had anti-BRSV titers of  $\leq 1:8$  as determined by indirect hemagglutination.<sup>20</sup> A bovine isolate of RSV, strain 375, inoculum was prepared as two batches from primary ovine fetal turbinate cells.<sup>18</sup> Treated sheep (n=16) received BRSV strain 375, ninth and tenth passages or BRSV strain 375, twelfth and thirteenth passages. Both batches of inoculum had a tissue culture infective dose<sub>50</sub> (TCID<sub>50</sub>) of  $1 \times 10^{6.7}$ /ml. Inoculum for control sheep (n=5) was prepared from either noninfected ovine fetal turbinate cells or from viral inoculum inactivated by heating to 56°C for 30 minutes. Sheep were anesthetized with xylazine, 0.1 mg/kg i.v. and positioned in right lateral recumbency. In order to expose the entire respiratory tree the right cranial lung lobe was instilled with 75 ml of inoculum using a fiberoptic bronchoscope. Additionally, each animal was inoculated transtracheally (20 ml) and intranasally (2 ml).

Two infected animals were killed at each of the following times: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 days P.I. Three control sheep were inoculated with sterile inoculum. Two were killed at 0.5 days P.I. and one at 3 days P.I. Another two control sheep received inactivated inoculum and were killed at 0.5 days P.I. Anti-RSV titers were determined on sera collected from each sheep prior to inoculation and at the time of euthanasia.<sup>20</sup>

Sheep were euthanatized with Sleepaway (Fort Dodge Labs, Fort Dodge, IA) and exsanguinated. Thoracic cavities were opened and tissues aseptically collected from the ventral apex of the right cranial lobe of the lung for isolation of virus<sup>18</sup> and bacteria. Bacterial isolation was performed on blood, MacKoney and Hayflick agar.

After sampling the cut apical surface of the lung was clamped with a hemostat. The thoracic viscera was removed from the thorax and the right cranial lobe of the lungs was fixed by intratracheal instillation of 4% paraformaldehyde in 0.1M cacodylate buffer at a pressure of 30 cm H<sub>2</sub>O. Tissues were fixed for 2-6 hours, trimmed, and stored in 0.1M cacodylate buffer.

For light microscopic evaluation, the cranial lobe of the right lung was divided into quadrants (cranial dorsal, cranial ventral, caudal dorsal, caudal ventral). A 1-2 cm<sup>2</sup> block was taken from approximately the same area within each quadrant from each animal, with the exception of one animal at 0.5 day P.I. in which only the caudal quadrants were sampled. Tissues were processed by routine paraffin embedding techniques. Nasal turbinates, trachea, right cranial bronchial and mediastinal lymph nodes, liver and spleen were also collected for histologic evaluation. These tissues were fixed by submersion in 4% paraformaldehyde in 0.1M cacodylate buffer overnight, stored in fixative and processed by routine paraffin techniques.

For all the tissues collected paraffin sections of 4  $\mu$ m thickness were stained with hematoxylin and eosin for histologic



evaluation. For the respiratory tract tissues and lymph nodes, additional sections were stained for BRSV antigen<sup>22</sup> and Factor VIII-related antigen<sup>3</sup> using an immunoperoxidase ABC procedure. The primary antibody against BRSV antigen was rabbit antisera made to the whole virus (Dr. C. Kucera, Norden Lab., Inc., Lincoln, NE). The primary antibody against Factor VIII related antigen was a rabbit antisera obtained commercially (DAKO Corp., Carpinteria, CA). The remaining immunoperoxidase reagents were obtained commercially (Vectastain ABC Kit, Vector Lab., Burlingame, CA).

Negative controls for immunoperoxidase procedure consisted of substitution of normal rabbit sera for the primary antibody. For BRSV specificity, additional negative controls consisted of lung tissue from noninfected sheep and lung tissue from sheep infected with either parainfluenzavirus 3 or ovine adenovirus. The parainfluenzavirus 3 and adenovirus infected tissues were obtained from previous experiments.

The following categories were created for scoring the number of infected cells in histological sections: turbinate epithelium, tracheal epithelium, bronchial epithelium, bronchiolar epithelium, Type I pneumocytes, Type II pneumocytes, alveolar macrophages, unidentifiable cells. When Type I pneumocytes on adjacent sides of septa stained for viral antigen, it was presumed to be the same cell and thus scored as 1. Each section was examined in its entirety.

For electron microscopic evaluation, the cranial lobe of the right lung was divided into cranial and caudal portions. From each portion, 6 or more samples were taken from tissue adjacent to areas samples for

light microscopy. The tissues were stored in 0.1M cacodylate buffer, post-fixed in osmium tetroxide, cleared in propylene dioxide, dehydrated through ethanol and embedded in epoxy resin in BEEM capsules (Electron Microscopic Services, Inc., Fort Washington, PA). One  $\mu\text{m}$  thick sections were cut and stained with toluidine blue. Thin sections were cut from selected blocks with a LKB ultramicrotome (LKB Instruments, Inc., Schaumburg, IL) and stained with lead citrate and uranyl acetate<sup>34</sup> for subsequent examination on a Phillips 410 electron microscope (Phillips Corp., New York, NY).

#### In vitro

Noninfected sheep were euthanatized with Sleepaway, (Fort Dodge Labs, Fort Dodge, IA) and exsanguinated. Lungs were removed aseptically and lung slices of 300  $\mu\text{m}$  thickness were obtained utilizing a Vibratome-1000<sup>R</sup> (Electron Microscopy Services, Redding, CA).<sup>23</sup> Slices were flooded with BRSV inoculum at a titer of approximately  $1 \times 10^6 \text{TCID}_{50}$ . To assess the value of lung slice organ cultures in studying viral respiratory pathogens, additional slices were inoculated with either ovine adenovirus, serotype 6, strain 151 at a titer of  $1 \times 10^{5.25} \text{TCID}_{50}$  or parainfluenzavirus-3 at a titer of  $1 \times 10^{5.75} \text{TCID}_{50}$ . Inoculum was left in contact with the slices for 1 hour. The slices were then rinsed with fresh minimal essential media supplemented with 10% fetal calf sera, 0.24 mg/ml of glutamine, and 0.05 mg/ml of gentomycin and slices were incubated at 37°C with 5%  $\text{CO}_2$  in room air.<sup>23</sup> Noninoculated control slices

were incubated concurrently. Control and infected tissue samples were taken at each of the following time periods: 0, 0.5, 1.0, 1.5, 2 and 3 days P.I. Slices were rinsed in phosphate buffered saline, fixed by submersion in 4% paraformaldehyde in 0.1M cacodylate buffer and processed for light microscopic evaluation. Ovine adenovirus and BRSV antigen detection was carried out using the indirect immunoperoxidase technique previously described. Parainfluenzavirus-3 antigen detection was carried out using direct immunoperoxidase with protease digestion to unmask viral antigen.<sup>22</sup> Rabbit anti-ovine adenovirus and biotinylated goat anti-human parainfluenzavirus-3 (Virostat, Portland, ME) antibodies were used for the primary antibodies against the respective viruses. Negative controls consisted of noninfected tissues and substitution of normal sera for primary antibody. Positive controls consisted of lung tissues from sheep which had been experimentally infected with the respective viruses.

## RESULTS

### In vivo

#### Clinical signs, serology, virus isolation

Clinical signs of respiratory disease were not observed until 6 and 7 days P.I. when two sheep presented with mild dyspnea. Rectal temperature was elevated ( $41^{\circ}\text{C}$ ) in one sheep at P.I. day 8. Starting in sheep killed at 4 days P.I. serum anti-BRSV titers were increased from preinoculation titers (Table 1). None of the control animals developed elevated anti-BRSV titers or had clinical signs. Respiratory syncytial virus was isolated from lung tissue of all sheep killed through 4 days P.I. and from one sheep killed at 6 days P.I. Virus was not isolated from lung tissues of BRSV inoculated sheep that were killed at 8 days P.I. Virus was not isolated from any control sheep. Bacteria, including mycoplasma, were not isolated from lung tissue of any of the sheep in this experiment.

#### Macroscopic lesions

There were locally extensive raised areas on the lung surface of one sheep killed at each 0.5 days P.I. and one killed at 1.0 day P.I. One of the sheep killed at 4 days P.I. had a minimally accentuated lobular pattern and the second sheep had a moderately enlarged cranial bronchial lymph node. One of the sheep killed at 6 days P.I. had a minimally accentuated lobular pattern. The lungs of the other sheep killed at 6 days P.I. failed to collapse and lung distention during

instillation of fixative was not uniform. Mediastinal lymph nodes of both sheep killed at 6 days P.I. were reddened. One sheep killed at 8 days P.I. had a focal area of pulmonary hemorrhage (<1 cm) and numerous raised lobules. The other sheep killed at 8 days P.I. also had multiple raised areas and the entire lung failed to completely collapse. Two control sheep, inoculated with sterile inoculum had focal reddened areas of atelectasis at 0.5 days P.I.

#### Microscopic lesions

Sheep killed at 0.5 day P.I. had minimal multifocal bronchiolitis and pneumonia with exudates of mixed populations of neutrophils, macrophages and lymphocytes and occasional areas of thickened alveolar septae (Figure 1). Tissues from sheep killed at 1.0 and 1.5 days P.I. had minimal to moderate multifocal bronchiolitis and pneumonia with exudate composed of neutrophils, macrophages and lymphocytes and sloughed epithelium in alveolar spaces and terminal bronchioles. Interstitial changes consisted of perivascular and peribronchiolar lymphocytic infiltrates and multifocal moderate distention of alveolar septae (Figure 2). At 2 days P.I. there was minimal multifocal bronchiolitis and interstitial pneumonitis with perivascular and peribronchiolar lymphocytic infiltrates. Some terminal bronchioles were partially occluded with mixtures of mucus and cellular exudate. There was focal necrosis of alveolar epithelium characterized by loss of lining epithelium, fibrin accumulation within alveolar septae and fibrin and RBCs in alveolar spaces pneumonia (Figure 3). At 3 days

P.I. there were multifocal mild peribronchiolar and moderate perivascular lymphocytic infiltrates, slight Type II cell hyperplasia, interstitial edema, and alveolar exudates comprised primarily of macrophages and lymphocytes (Figure 4). Histological changes at 4, 6 and 8 days P.I. were similar to the changes in lung tissue of sheep killed at 3 days P.I., with extension of the perivascular and peribronchiolar lymphocytic infiltrates into distended alveolar septa (Figure 5). Multifocally, terminal bronchioles lined by necrotic epithelium characterized by pyknotic nuclei and vacuolated cytoplasm. There were also areas of attenuated bronchiolar epithelium at the latter time periods. Exudates of macrophages, lymphocytes and neutrophils were in bronchioles and alveoli. Alveolar exudates were composed primarily of lymphocytes and macrophages.

In tissues other than lung the following was seen. Starting at 1.0 day P.I., cranial bronchial and mediastinal lymph nodes were minimally edematous with prominent lymphoid nodules, and increased numbers of macrophages and lymphocytes were present within medullary sinuses. Turbinates and trachea of some animals had very minimal submucosal lymphocytic infiltrates. One animal had focal epithelial erosion in the trachea. There were no significant changes in any other tissues from infected animals.

Histological changes in control animals at 0.5 day P.I. included minimal, bronchiolitis and pneumonia with exudate consisting primarily of neutrophils with fewer macrophages and lymphocytes. There were also minimal multifocal perivascular lymphocytic infiltrates and very

minimal Type II cell hypertrophy. Histological changes at 3 days P.I. included scattered minimal perivascular and peribronchiolar lymphocytic infiltrates.

#### Immunohistochemistry

At 0.5 days P.I., a few macrophages were the only cells positive for BRSV antigen (Table 2). At 1 day P.I. there was viral antigen in Type I pneumocytes, bronchiolar epithelium, alveolar macrophages and a single bronchial epithelial cell. At 1.5 and 2 days P.I. antigen was present in Type I pneumocytes, macrophages and bronchiolar epithelium. At the remaining time periods, in addition to the above cell types, antigen was detected in Type II pneumocytes (Table 2). The staining pattern up through 3 days P.I. was one of a few scattered infected cells per high power field and the foci of infection were often not associated with inflammatory cells (Figures 6, 7, 8 & 9). At 6 days P.I. there were locally extensive areas of bronchiolar and alveolar epithelium which stained for antigen (Figures 10 & 11). Antigen was often concentrated at the apical surface of ciliated and non-ciliated epithelial cells of bronchioles. At 8 days P.I., all tissues examined were negative for antigen. Some of the cells which stained for BRSV antigen resembled endothelial cells. However, the cells that appeared to be endothelium and stained for viral antigen lacked staining for Factor VIII antigen in adjacent serial sections. Occasionally, non-specific staining was localized along the area of the basement membranes of bronchiolar and alveolar epithelium. There was also

staining of some debris in alveolar spaces and bronchiolar lumens. There was no staining for viral antigen in any of the bronchial or mediastinal lymph nodes.

#### Electron microscopy

Virions were identified ultrastructurally in association with bronchiolar and alveolar epithelium (Figures 12 & 13). Despite several trials of many different protocols including etching of sections, pre-embedding labeling of tissues, and resin removal from sections, viral antigen was not detected at the electron microscopic level using immunocytochemical techniques.

#### In vitro

There was staining for parainfluenzavirus-3 antigen in macrophages at 0.5 days P.I. At 1 day P.I. there was staining for parainfluenzavirus-3 antigen in bronchiolar epithelium. At 3 days P.I. bronchiolar epithelium in every airway and occasionally alveolar epithelium of inoculated lung slices stained for parainfluenzavirus-3 antigen. There was no staining of BRSV or ovine adenovirus antigen in any of the lung slices inoculated in vitro.



## DISCUSSION

The paucity of BRSV positive macrophages, in comparison to other infected cell types, is consistent with sheep alveolar macrophages not being very susceptible to BRSV infection in vitro.<sup>33</sup> The lack of positive staining in animals given inactivated inoculum indicates that macrophage staining most likely represents infection and not staining of phagocytized viral antigen. Alveolar macrophages are a heterogenous population.<sup>26</sup> In vitro, human monocytes are more supportive of RSV replication as they age and become more macrophage-like.<sup>16</sup> The alveolar macrophages staining for BRSV in these sheep may represent a sub-population that is permissive in regards to BRSV replication. Alveolar macrophages were the first cells in which BRSV antigen was detected. For alveolar macrophages to attain detectable levels of viral antigen before any other cells in the lung indicates that either viral replication is more rapid in alveolar macrophages than in epithelium or that the rate of exposure to the virus is higher for alveolar macrophages than for epithelium. There are no reports comparing the replication of BRSV in macrophages to that in epithelium. The exposure rate of alveolar macrophages to BRSV would be expected to be higher than the exposure rate of respiratory epithelium because macrophages are not provided protection by mucus or alveolar lining material.

Our results are consistent with the previous studies in sheep, in which BRSV infection produced a bronchiolitis, interstitial pneumonitis

and pneumonia. Viral antigen was detected at 2 days P.I. primarily in bronchiolar and alveolar epithelium.<sup>8,32</sup> In experimentally infected calves BRSV antigen was detected at 2 days P.I. at all levels of the respiratory tract but was most prevalent in the bronchi.<sup>6,7,31</sup> In our study, the staining of antigen in both bronchiolar and alveolar epithelium at 1 day P.I. suggests that there is not a preferential primary infection of one of these epithelial cell types. Viral antigen positive cells that histologically resembled endothelium but failed to stain for Factor VIII-related antigen in serial sections probably represent Type I pneumocytes lining narrow alveolar spaces. The paucity of infected cells and inability to label viral antigen at the electron microscopic level precluded us from carrying out morphometric analysis on infected cell types. However, the light microscopic data indicate that the greatest proportion of infected cells are Type I pneumocytes. Type I pneumocytes cover the majority of lung surface area and so have a greater risk of exposure in comparison to other cell types in the lung. The number of each of the different cell types infected could reflect the incidence of exposure and not a preference of infection.

The tremendous capability of the lung to clear itself of airborne pathogens was indicated by the paucity of BRSV-infected cells in these sheep which had been infected by flooding an entire lung lobe with viral inoculum. The mucociliary apparatus comprises a large portion of the lungs' defense capabilities.<sup>11</sup> Mucus and alveolar lining material prevent airborne pathogens from gaining access to the

underlying epithelium.<sup>10</sup> However, the protection is not uniform throughout the respiratory tract. The mucous layer varies in composition along the respiratory tract and may not be continuous.<sup>10,11</sup> In addition, mucus and alveolar lining material are cleared more slowly from the terminal airways and alveoli than from the bronchi and trachea. The slower clearance favors the establishment of many respiratory infections in the distal respiratory tract.<sup>10</sup> Bovine respiratory syncytial virus is able to infect a number of different cell types in vitro<sup>21</sup> and in our experiment was present in bronchial epithelium in one sheep. This suggests that the epithelium of the upper respiratory tract would support viral replication if exposed. The restriction of BRSV infection to bronchioles and alveoli in this study is indicative of the areas of the respiratory tract that are least protected.

The failure of antigen detection and virus isolation at 8 days P.I. coincided with a marked seroconversion, as such virus may have been cleared or masked by immunoglobulins.<sup>2</sup> This elimination of virus plus minimal to moderate pulmonary lesions is evidence of protection provided by the immune response in these sheep.

The paucity of BRSV infected cells in lung tissues from inoculated animals was unexpected. This could explain the lack of infection in the lung slices after in vitro inoculation. A low incidence of infection combined with the limited number of cells in a lung slice might not allow establishment of infection. The validity of using lung slices for studying some viral infections was evidenced by the

infection of slices by parainfluenzavirus-3. The comparable titers of the 3 viruses used in this study indicates that parainfluenzavirus-3 is the most capable of establishing pulmonary infections in our model.

The use of our sheep model allowed us to determine the site of infection initiation and progression. All previous experimentation with BRSV in calves and sheep has used either aerosolization,<sup>32</sup> or transtracheal inoculation.<sup>6,7,8,31</sup> These forms of inoculation give a very limited exposure of the lung surface area to the virus. To determine the initial cells infected by BRSV we had to ensure that the tissues collected at 0.5 and 1.0 day P.I. had been exposed to the virus. By flooding a lobe of the lung we ensured exposure of the collected lung tissue and we were able to detect BRSV-infected cells before any secondary foci of viral infections could be established. Using our model, comparison of antigen staining and lesions of BRSV infections in cattle and sheep may help explain the pathogenesis of severe respiratory disease caused by BRSV infection in cattle. In addition, attempts can be made to enhance the lesions caused by BRSV infection of sheep; for example, by inoculating sheep of different ages or serostatus with respect to BRSV.

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Table 1. Serology and virus isolation at various times post-inoculation for BRSV-infected and control sheep

Sheep No.	Time post-inoculation days	Pre-inoculation <sup>a</sup> anti-BRSV IHA titer	Post-inoculation <sup>b</sup> anti-BRSV IHA titer	BRSV isolation
<u>Infected Sheep</u>				
2829	0.5	1:4	1:4	+
2878		1:4	1:4	+
2802	1.0	1:2	1:4	+
2796		1:4	1:4	+
2814	1.5	1:4	1:4	+
2841		1:4	1:4	+
2801	2.0	1:4	1:4	+
2870		1:4	1:4	+
2799	3.0	1:4	1:4	+
2805		1:4	1:4	+
2825	4.0	1:8	1:512	+
2877		1:4	1:16	+
2942	6.0	1:4	1:4	-
2951		1:4	1:8	+
2962	8.0	1:4	1:1024	-
2978		1:4	1:1024	-

Control Sheep

Sterile inoculum

2828	0.5	1:2	1:2	-
2824	0.5	1:4	1:4	-
2830	3.0	1:4	1:4	-

Inactivated inoculum

2815	0.5	1:4	- <sup>c</sup>	-
2865	0.5	1:8	- <sup>c</sup>	-

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<sup>a</sup>Pre-inoculation sera was collected on the day of inoculation.

<sup>b</sup>Post-inoculation sera was collected at the time of euthanasia.

<sup>c</sup>Post-inoculation blood samples were not taken.

+ Virus was isolated from lung tissue.

- No virus was isolated from lung tissue.

Table 2. Cell types staining for BRSV antigen at various times post-inoculation in infected sheep<sup>a</sup>

Sheep No.	P.I. <sup>b</sup> days	Bronchial epithelium	Bronchiolar epithelium	Type I pneumocytes	Type II pneumocytes	Alveolar macrophages	Other
2829	0.5	0	0	0	0	1	0
2878	0.5	0	0	0	0	2	0
2802	1.0	1	2	12	0	6	2
2796	1.0	0	4	0	0	5	1
2814	1.5	0	12	11	0	6	1
2841	1.5	0	6	18	0	1	9
2801	2.0	0	5	14	0	2	3
2870	2.0	0	15	17	0	9	10
2799	3.0	0	20	141	6	1	28
2805	3.0	0	9	157	2	0	7
2825	4.0	0	16	21	1	3	4
2877	4.0	0	345	446	29	1	56
2942	6.0	0	416	146	2	3	86
2951	6.0	0	7	43	3	0	32
2962	8.0	0	0	0	0	0	0
2978	8.0	0	0	0	0	0	0

<sup>a</sup>Turbinate and tracheal epithelium had no staining.

<sup>b</sup>Days post-inoculation.

Figures 1-5. BRSV infected lung tissue. Hematoxylin & eosin.

Figure 1. Lung, sheep #2878, 0.5 day P.I. Mixed inflammatory cells within bronchioles and alveolar spaces with scattered areas of thickened alveolar septae. Hematoxylin and eosin. X100

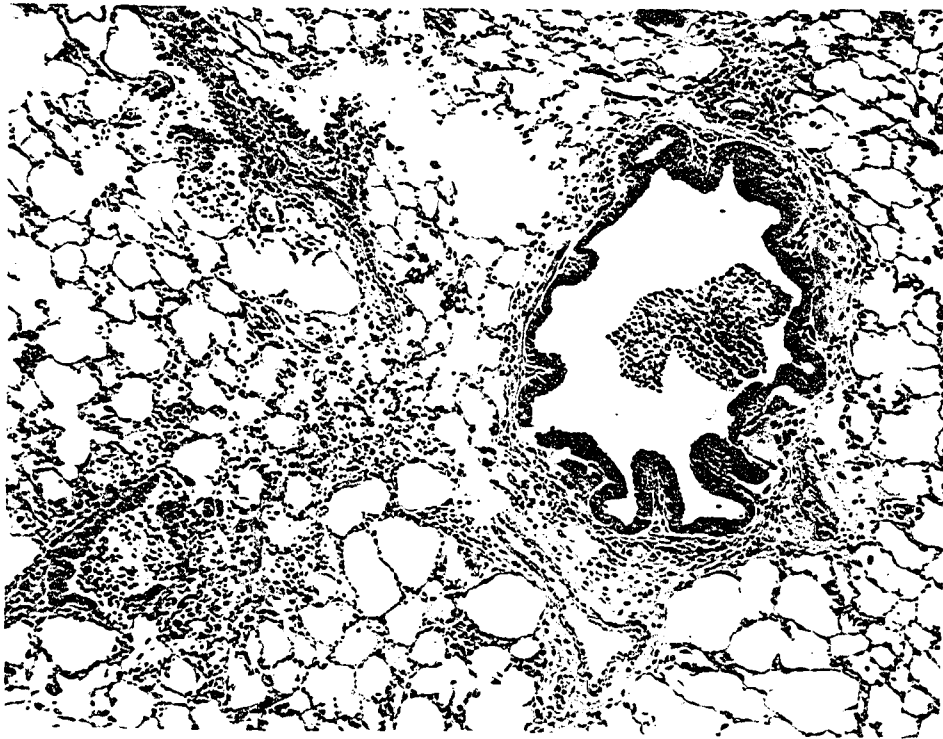


Figure 2a. Lung, sheep #2814, 1.5 day P.I. Mixed inflammatory exudate within, bronchi, terminal bronchioles and alveoli. Multi-focal paribronchiolar and perivascular lymphocytic infiltrates. Hematoxylin and eosin. X40

Figure 2b. Lung, sheep #2841, 1.5 day P.I. Peribronchiolar and perivascular lymphocytic infiltrate with sloughed bronchiolar epithelium (arrow) and interstitial edema. Hematoxylin and eosin. X195



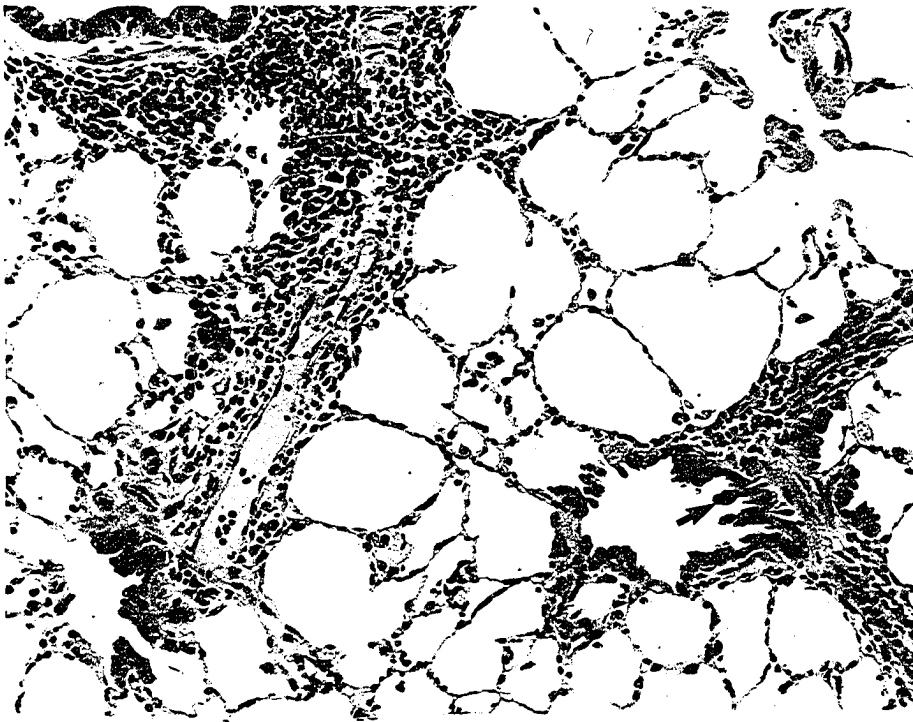
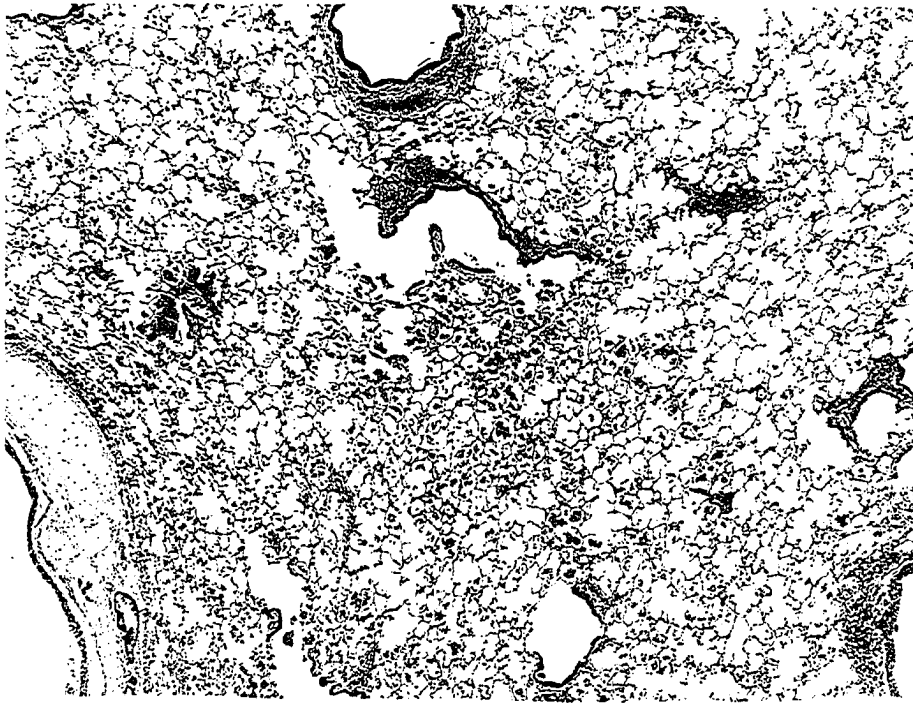


Figure 3. Lung, sheep #2870, 2 days P.I. Fibrin (arrow) and RBCs (arrowhead) in alveolar spaces with low numbers of mixed inflammatory cells. Hematoxylin and eosin. X262

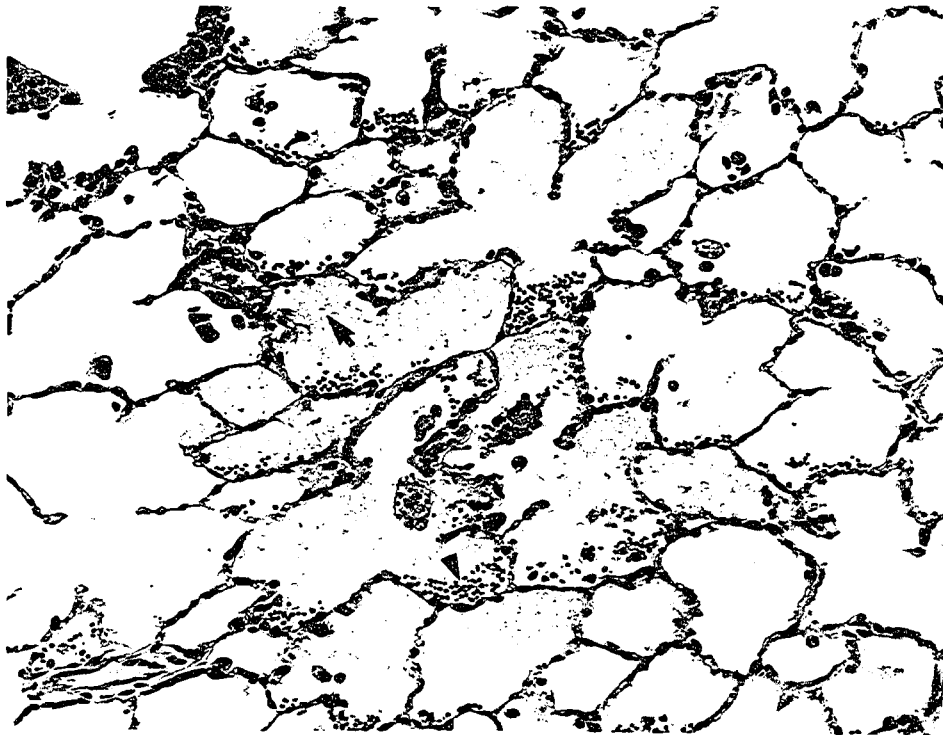


Figure 4a. Lung, sheep #2805, 3 days P.I. Perivascular lymphocytic infiltrates with perivascular edema (arrow). Hematoxylin and eosin. X50

Figure 4b. Lung, sheep #2799, 3 days P.I. Alveolar septal distention with interstitial infiltrates of macrophages and lymphocytes. Minimal Type II pneumocyte hyperplasia. Alveolar exudate of macrophages, lymphocytes and lesser numbers of neutrophils. Hematoxylin and eosin. X195

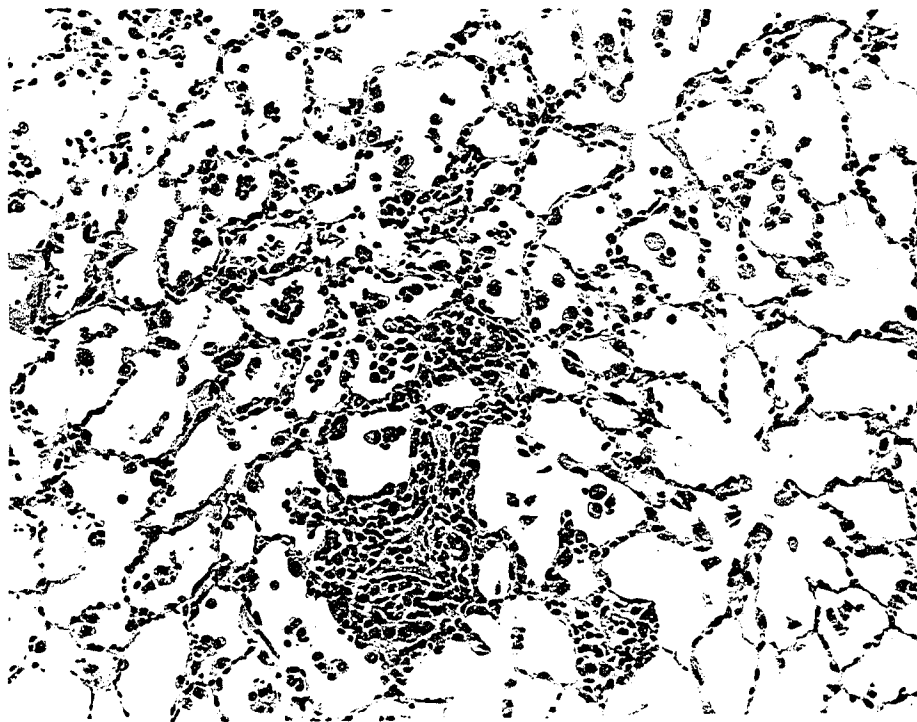
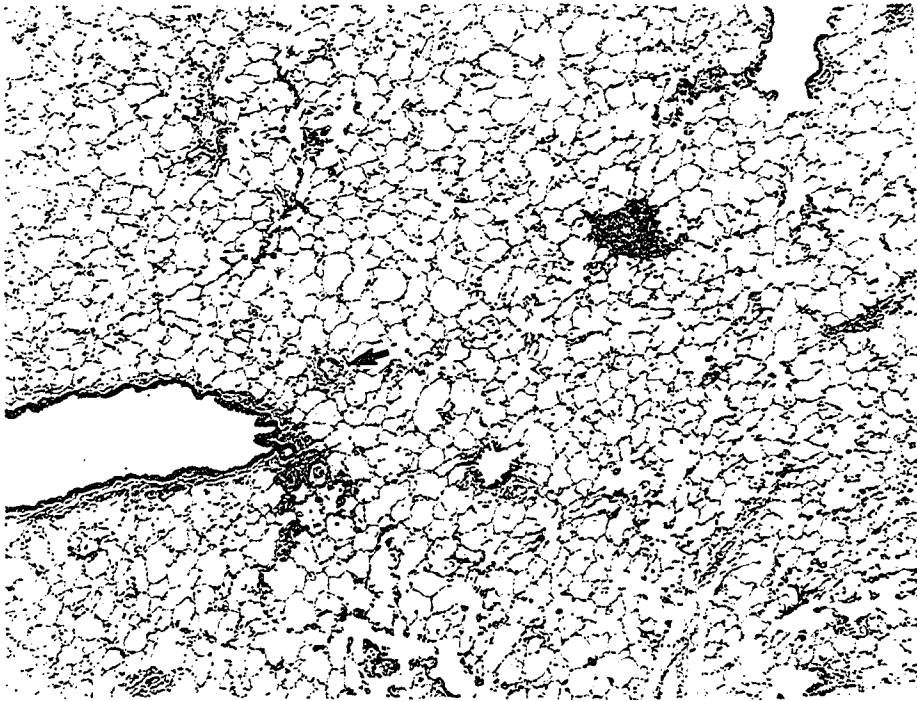
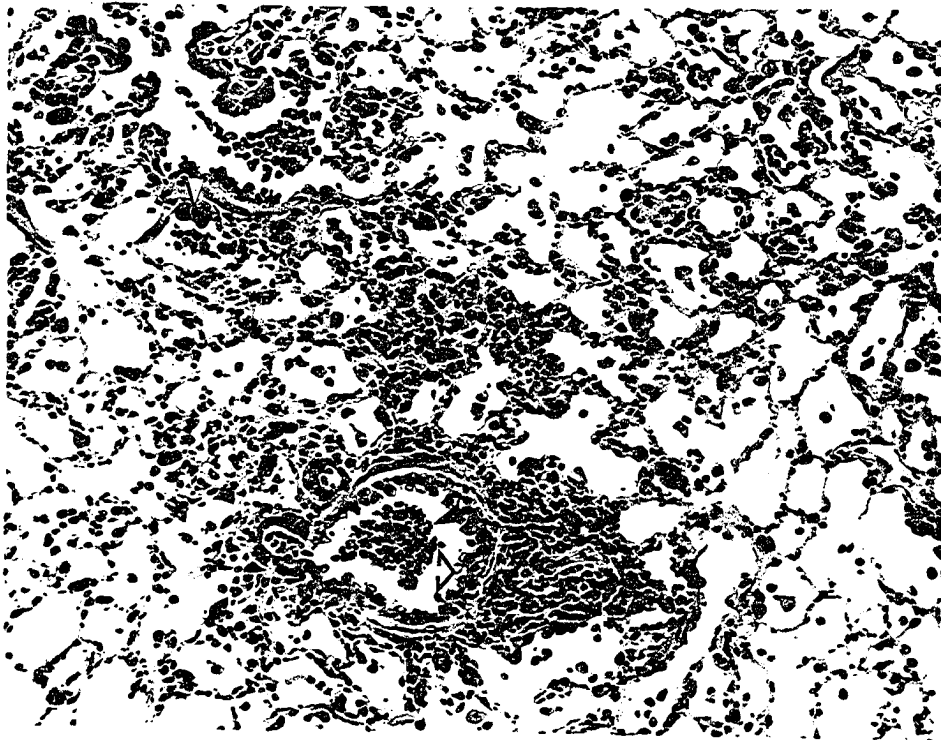


Figure 5. Lung, sheep #2942, 6 days P.I. Inadequately instilled area as result of bronchiolar plugs formed from necrotic cell debris and inflammatory cells (arrow). Peribronchiolar lymphocytic infiltrates extend into the alveolar septa. Some alveolar spaces are filled with macrophages, lymphocytes and lesser numbers of neutrophils. Type II pneumocytes are hyperplastic (arrowheads, closed), bronchiolar epithelium is irregular and vacuolated (arrowhead, open). Hematoxylin and eosin. X156



Figures 6-12. Lung tissue stained with immunoperoxidase for BRSV antigen. Infected cells stain brown. Mayer's hematoxylin counterstain.

Figure 6. Lung, sheep #2801, 2 days P.I. BRSV infected Type I pneumocyte outlines the corner of an alveolus. X590

Figure 7. Lung, sheep #2799, 3 days P.I. BRSV infected Type I and Type II pneumocytes. X200



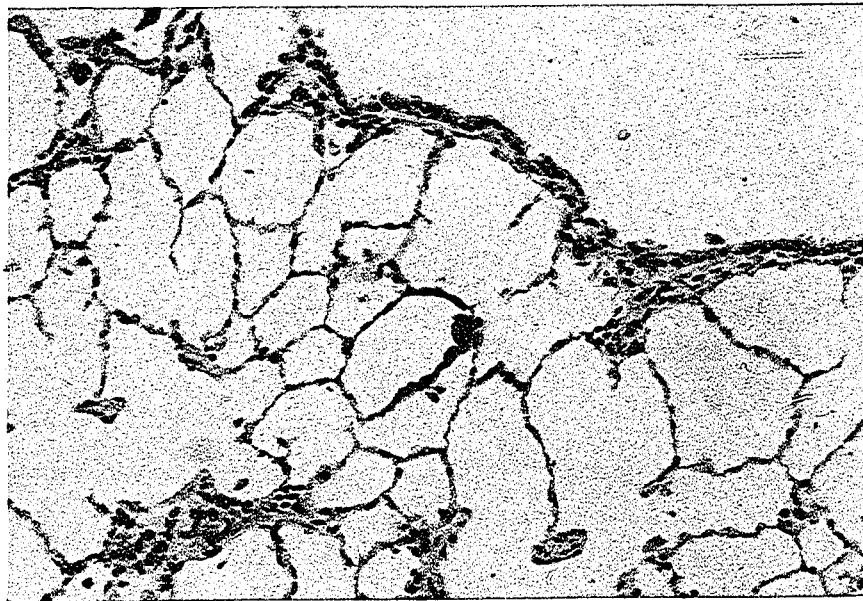
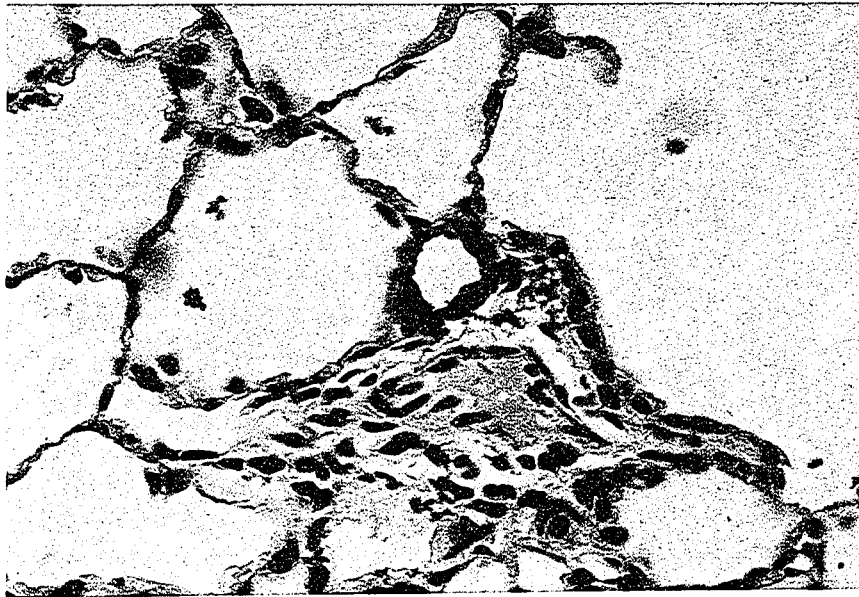


Figure 8. Lung, sheep #2799, 3 days P.I. BRSV infected bronciolar cells, intact (arrow) and partially sloughed (arrowhead) with associated macrophage. Note apical portion of cells staining for antigen. X500

Figure 9. Lung, sheep #2799, 3 days P.I. Necrotic Type I pneumocyte (arrow) and unidentifiable cell (arrowhead) stain diffusely for BRSV antigen. X630

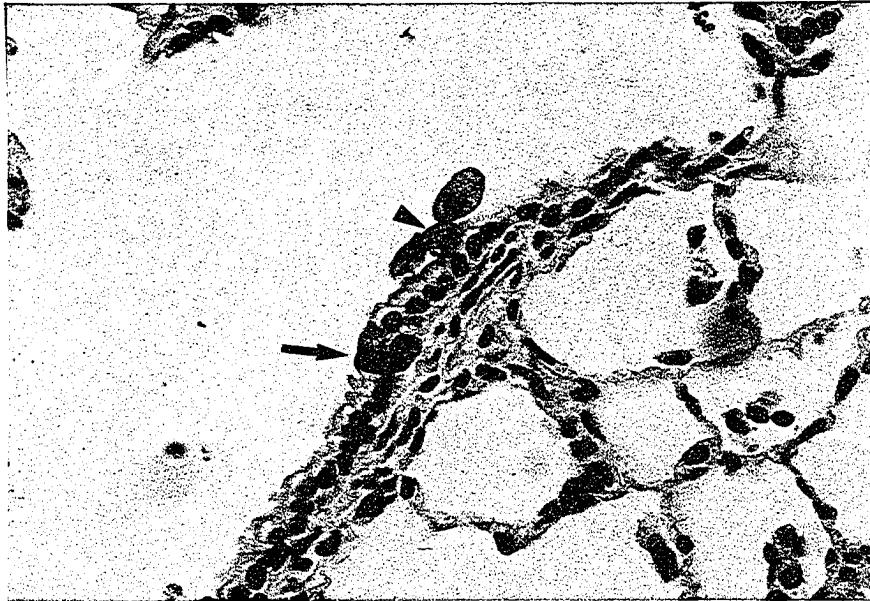
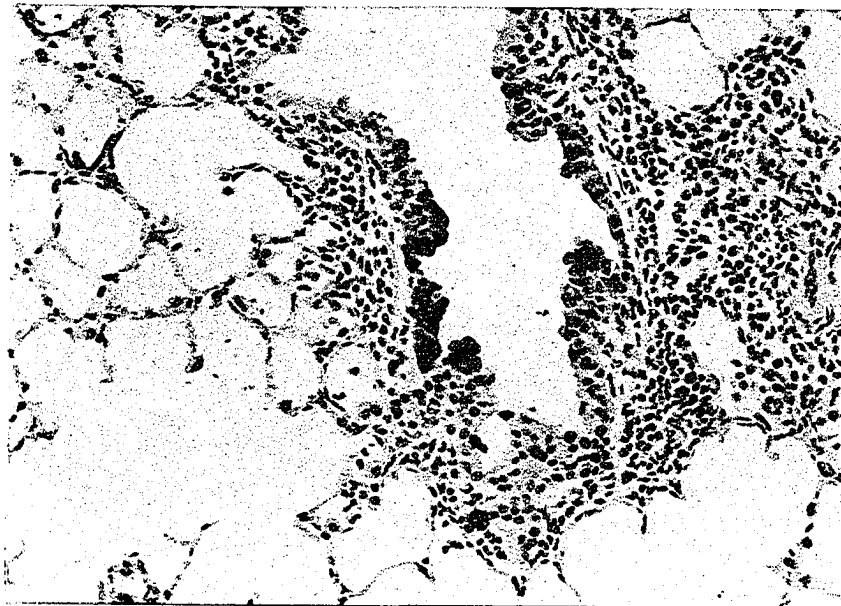
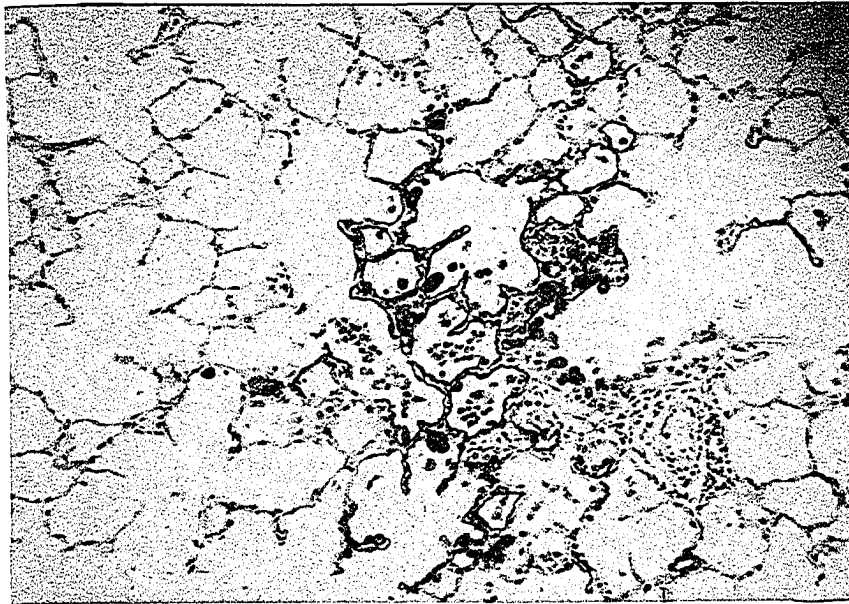


Figure 10. Lung, sheep #2877, 4 days P.I. Locally extensive area of BRSV infected Type I, Type II, and bronchiolar epithelium. X128

Figure 11. Lung, sheep #2942, 6 days P.I. Infected epithelium lines portions of a bronchiole. X200

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Figures 12 & 13. BRSV infected lung tissue.

Figure 12a. Lung, sheep #2801, 2 days P.I. BRSV  
particles associated with ciliated bronchiolar  
epithelium. Both cells are partially sloughed.  
Bar = 1  $\mu$ m

Figure 12b. Lung, sheep #2801, 2 days P.I. Same field as Figure 12a,  
higher magnification of viral particles (arrows). Bar = 1  
 $\mu$ m

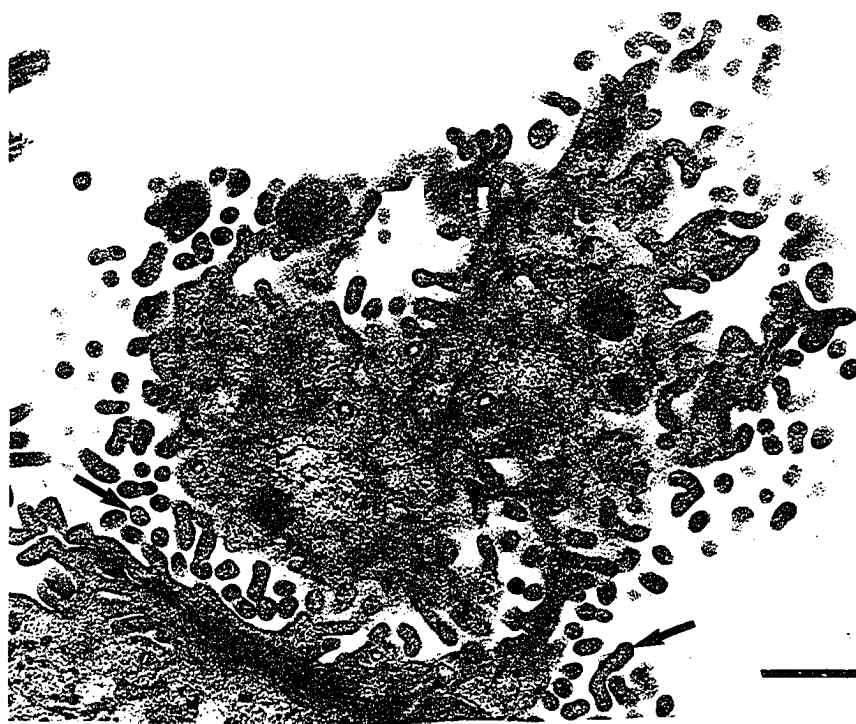
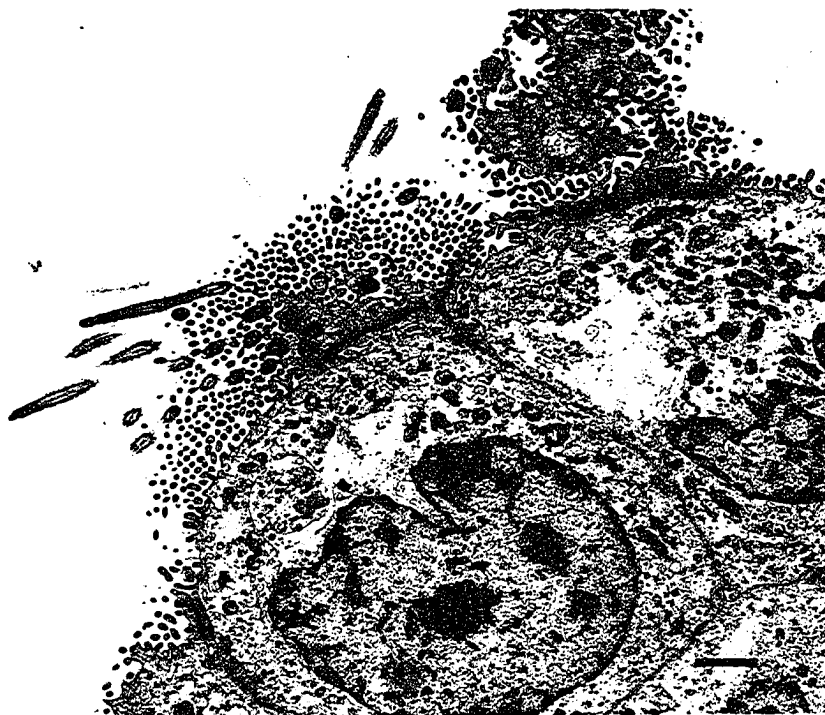
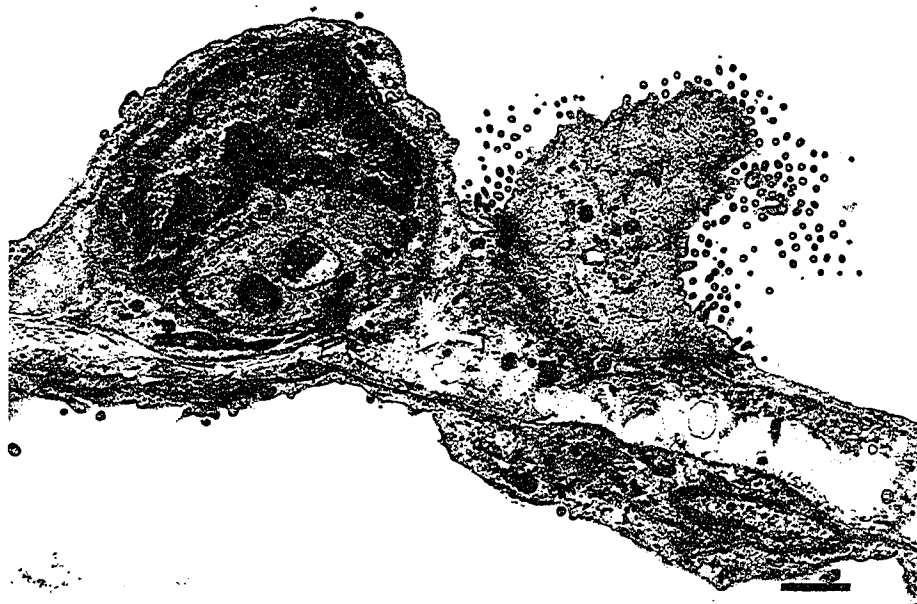


Figure 13a. Lung, sheep #2841, 1.5 days P.I. BRSV particles and buds in association with an unidentified alveolar epithelial cell. Bar = 1  $\mu$ m

Figure 13b. Lung, sheep #2841, 1.5 days P.I. Same field as Figure 13a, higher magnification of infected cell and associated viral particles (arrow). Bar = 0.5  $\mu$ m

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## GENERAL DISCUSSION AND SUMMARY

There are several reports of bronchitis, interstitial pneumonitis and pneumonia caused by experimental BRSV infection in calves and lambs.<sup>15,17,25,43,87,89</sup> All but one of the reported studies involve neonatal, colostrum deprived or gnotobiotic animals.<sup>89</sup> Natural infection of calves with BRSV can cause atypical interstitial pneumonia.<sup>5,29</sup> This cannot be reproduced experimentally. Establishment of infection is a critical step in any infectious process. Bovine respiratory syncytial virus antigen has been detected at 2 days P.I. in the lung tissue of experimentally infected calves and lambs.<sup>15,17,87,89</sup> Tissues collected from calves at 1 day P.I. were examined but no viral antigen was detected.<sup>15</sup> Within a 2-day time period BRSV could have replicated and spread to establish secondary foci of infection.<sup>65</sup>

The goals of this project were to determine the cell type(s) in the lung that first stained for BRSV antigen after exposure to BRSV and to follow progression of the infection. To assist in obtaining these goals the following techniques were developed; a lung organ culture technique that allowed viable lung tissue to be maintained in vitro for 4 days and an immunoperoxidase staining technique that allowed detection of BRSV antigen in sheep lung tissues that had been processed by routine paraffin techniques. Bovine respiratory syncytial virus infected cells were identified in lung tissue from sheep which

had been inoculated with BRSV by flooding the right cranial lobe of the lung with virus and from lung slice organ cultures which had been inoculated with BRSV in vitro. These inoculation techniques were used to optimize the possibility that the sampled lung tissue would contain infected cells and to infect large numbers of cells so morphometric analysis could be performed to determine if a cell type was preferentially infected. We were not attempting to produce severe respiratory disease with BRSV infection in lambs.

We chose to use conventionally reared yearling lambs because RSV is a natural respiratory pathogen in sheep and we want to pursue using BRSV infection in lambs as a model to study RSV infection in cattle and humans.

In the first portion of this study, an immunoperoxidase staining technique was developed to detect BRSV antigen in fixed, paraffin embedded tissues. Respiratory syncytial virus antigen is masked by routine fixation and processing procedures. Previous studies of calves and lambs experimentally infected with BRSV have detected viral antigen using immunofluorescence on frozen sections<sup>15,17,87,89</sup> or immunoperoxidase on fixed tissue sections after enzymatic digestion.<sup>13</sup> Both of these techniques sacrifice structural integrity for antigen detection. In our immunoperoxidase technique, a detergent was used to unmask BRSV antigen in fixed tissue. This allowed detection of BRSV antigen without sacrifice of structural integrity and in addition decreased non-specific background staining in comparison to other techniques.

In the second portion of this project, the specific cell types which stained for BRSV antigen was determined for lung tissue from infected sheep and for lung tissue infected in vitro. The first cells to stain for BRSV antigen were alveolar macrophages in tissues from sheep killed at 0.5 day P.I. The majority of staining for BRSV antigen, present from 1-6 days P.I., was within bronchiolar epithelium and Type I pneumocytes. Type II pneumocytes stained for BRSV antigen from 3-6 days P.I. With the exception of one bronchial epithelial cell there was no staining for viral antigen above the level of the bronchioles. Attempts failed to stain BRSV antigen with immunogold techniques. Virions were identified ultrastructurally in association with bronchiolar and alveolar epithelium. The lung organ culture technique allowed us to inoculate lung tissue with virus, in vitro and determine, using immunoperoxidase, the infected cell types. Lung organ cultures were shown to be useful for evaluating parainfluenzavirus-3 infection but not for BRSV or an ovine adenovirus infection. Parainfluenzavirus-3 readily infected lung slices in vitro. However, no BRSV or ovine adenovirus antigen was detected by immunohistochemistry in any of the lung slices inoculated in vitro.

The paucity of BRSV-infected cells and failure to detect viral antigen at the ultrastructural level with immunocytochemical techniques precluded morphometric analysis of infected cell types. The high numbers of infected bronchiolar epithelium and Type I pneumocytes may reflect incidence of exposure to BRSV and not a preferential infection of these two cell types.

The failure of BRSV to establish extensive infection in these sheep attests to the tremendous clearance capabilities of the lung.<sup>28</sup> The moderate lesions produced by BRSV infection in lambs is consistent with previous reports.<sup>25,89</sup> There are no reports of experimental BRSV infection in yearling cattle.

Sheep infected experimentally with BRSV can be used as a model to study severe respiratory disease caused by BRSV infection in cattle. Determining the pathogenesis of severe respiratory disease caused by BRSV infection will help minimize RSV's contribution to respiratory disease and provide knowledge of inflammatory processes in the lung which may apply to other acute respiratory disease processes.

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## Appendix A:

Use of the Vibratome® for producing uniform slices for  
lung organ culture

Adult BALB/C mice were killed by cervical dislocation and the thoracic cavity opened aseptically. Lungs and trachea were dissected free from surrounding tissues, removed from the thoracic cavity and kept moist with 0.01M phosphate buffered saline (PBS), pH 7.6, at 37°C. A mixture of 2% agarose (SeaKemp ME, FMC Bio Products, Rockland, ME) in minimal essential media (MEM) was prepared at 56°C. The mixture was supplemented with 10% fetal calf sera, 0.05 mg/ml Gentocin (Schering Corp., Kenilworth, NJ) and 0.24 mg/ml glutamine (supplemented MEM). This mixture of agarose and MEM was cooled to 37-40°C and transferred to a 5 ml syringe which was connected to the mouse trachea via an adapter. The lungs were gently instilled with the liquid agarose-MEM until they were uniformly distended. A second set of lungs, inflated to a pressure of 20 cm<sup>2</sup> H<sub>2</sub>O, was used as a guide to avoid overinflation. After instillation, the trachea was clamped and the lungs were placed in a sealed plastic bag and cooled at 4°C for 1/2 hour which allowed the gel to solidify. The lungs were then trimmed into 0.5 cm<sup>3</sup> cubes and 300 µm thick sections were cut at 4°C on a Vibratome (E.M. Supply Center, Redding, CA). The slices were stored at 4°C for less than 1.5 hour in supplemented MEM,

supplemented as described above, and then spread onto the surface of 0.2% agarose in supplemented MEM, which was contained in 24 well microtiter plates. Each well contained 1 ml of 0.2% agarose-media mixture. This concentration of agarose provided adequate support to maintain the section at the air-liquid interface. The tissues were incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub> in room air.

Incubated tissues were harvested daily for 5 days. After rinsing with PBS, tissues were fixed in 10% neutral buffered formalin for histological evaluation or in 2.5% glutaraldehyde in 0.01M cacodylate buffer at 4°C for ultrastructural evaluation. The only tissues examined with electron microscopy were those harvested at 3 days incubation.

Microscopic examination of the tissue after 0 to 4 days of incubation revealed that alveolar septa and perivascular spaces were diffusely distended and occasionally alveolar septa were fractured. Lymphocytes were present within the interstitium in low numbers. Through 4 days incubation the bronchioles were lined by ciliated cuboidal epithelium with occasional areas of attenuated epithelium. (Figure 1) At 5 days incubation there was extensive areas of necrotic bronchiolar and alveolar epithelium with pyknotic nuclei and granular cytoplasm. Many bronchioles were devoid of epithelium.

Use of the Vibratome® allows slices of uniform thickness (300 µm) to be obtained and maintained for 4 days in vitro without



histological evidence of degeneration. The slices are of adequate size to allow processing and subsequent histological examination of multiple airways and lung parenchyma within the same section. This lung organ culture technique allows the study of acute processes in intact lung tissue under very well defined in vitro conditions.

Figure 1. Lung tissue after 4 days incubation. Bronchiolar and alveolar epithelium show no histological signs of deterioration and cilia are evident (arrow). X250



## Appendix B:

## Protocols used in attempts to detect BRSV antigen at the electron microscopic level

- I. Reprocessing of histological sections for electron microscopic examination.
  - a) A histological section is stained for viral antigen using an immunoperoxidase technique
  - b) Mark on bottom of glass slide an area of tissue section that is positive for viral antigen.
  - c) Remove coverslip by soaking in xylene, 60°C for 20 minutes.
  - d) Trim away excess tissue, do not allow section to dry.
  - e) Clear and infiltrate by placing slide in Koplin jars.
    - i) xylene, 5 minutes.
    - ii) 50% propylene oxide in xylene, 5 minutes.
    - iii) propylene oxide, 5 minutes.
  - f) Cover tissue with 25% resin/75% propylene oxide mixture, let stand for 5 minutes, do not let tissue dry prior to infiltration.
  - g) Incubate for 10 minutes at 60°C .
  - h) Place inverted BEEM capsule (Electron Microscopic Services, Fort Washington, PA.) nearly full of resin over tissue.
  - i) Incubate in oven for 60-90 minutes at 95-100°C. .
  - j) Remove from oven and pop off inverted BEEM capsule containing tissue before slide cools.
  - k) Section and stain for transmission electron microscopy.

Results: Lack of adequate tissue preservation or extensive tissue damage incurred during reprocessing prevented any assessment of tissue ultrastructure.

II. Bovine respiratory syncytial virus antigen detection with immunogold labeling in epoxy embedded ovine fetal turbinate cells.

1.) Direct labeling technique.

- a) Respiratory syncytial virus infected cells were suspended by trypsinization, formalin fixed and embedded in 2% agarose.
- b) Tissue was routinely processed, infiltrated and embedded in epoxy resin.
- c) Sections of 70-90 nm thickness on nickel coated grids were rehydrated for 10 minutes with distilled water at room temperature.
- d) Etched by incubating<sup>a</sup> with saturated sodium metaperiodate for 5 to 10 minutes at room temperature.
- e) Rinsed 3 times for 5 minutes each with filtered distilled water at room temperature.
- f) Antigen was unmasked by incubating with filtered Tris-EDTA-Tween buffer<sup>b</sup> for 10 minutes at room temperature.
- g) Blocked with normal goat serum for 10 minutes at 37°C.
- h) Primary antibody (5 nm gold labeled anti-RSV monoclonal antibody) was applied and incubated overnight at 4°C.
- i) Jet washed with 20 ml of filtered Tris-EDTA-Tween buffer.
- j) Counterstained with uranyl acetate and lead citrate.

Negative controls consisted of substitution of normal rabbit sera for primary antibody.

Results: No gold was present on sections.

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<sup>a</sup> All incubations and rinses were carried out by floating grids, section side down, on a drop of reagent.

<sup>b</sup> 0.05M Tris buffered saline + 1mM EDTA + 0.05% Tween 20.

## 2.) Indirect labeling technique.

- a) Respiratory syncytial virus infected cells were suspended by trypsinization, formalin fixed, and embedded in 2% agarose.
- b) Tissue was routinely processed, infiltrated, and embedded in epoxy resin.
- c) Sections of 70-90 nm thickness on nickel coated grids were rehydrated for 10 minutes with distilled water at room temperature.
- d) Etched by incubating with saturated sodium metaperiodate for 5 to 10 minutes at room temperature.
- e) Rinsed 3 times for 5 minutes each with filtered distilled water at room temperature.
- f) Rinsed for 5 minutes with filtered TBS .
- g) Antigen was unmasked by incubating with Tris-EDTA-Tween + 1% BSA buffer for 10 minutes at room temperature.
- h) Block by flooding with undiluted sera from the species in which the secondary antibody was prepared (Normal goat sera)
- i) Excess serum was blotted off.
- j) Primary antibody (rabbit anti-RSV antibody) diluted in TBS was applied and incubated overnight at 4°C.
- k) Jet washed with 20 ml of Tris-EDTA-Tween buffer.
- l) Secondary antibody (15 nm goat labeled anti-rabbit antibody) diluted in TBS<sup>a</sup> was applied and incubated for 1 hour at 37°C.
- m) Jet washed with 20 ml of filtered TBS, 20 dips in 5 changes of filtered TBS then rinsed 3 times for 5 minutes each with filtered TBS at room temperature.
- n) Jet washed with water.
- o) Counterstained with uranyl acetate and lead citrate.

Negative controls consisted of substitution of normal rabbit sera for primary antibody.

**Results:** Specific labeling of antigen was not present. Gold particles were present over cellular and acellular portions of the sections. There was no increased concentration of gold particles on viral structures.

The lack of any increased concentration of gold particles on virions in the epon embedded sections suggested antigenic sites were unavailable either because of destruction during processing and etching or because of resin interference with antibody penetration. Epon resin is poorly penetrated by antibodies. The decision was made to try a hydrophobic resin (LR White) which allows better penetration of antibodies, thereby increasing antigen accessibility.

Immunogold with silver enhancement was chosen for a detection system because it would allow antigen detection at both the light and electron microscopic levels.

III. Immunogold-silver enhancement for detection of RSV antigen in paraformaldehyde fixed tissues embedded in LR White resin or paraffin .

- a) Tissues were processed, infiltrated and embedded in LR White<sup>a</sup> using thermal polymerization.
- b) 1  $\mu$ m LR White sections were cut on a glass knife and floated onto acid cleaned slides using a drop of water or acetone. Slides were heated on a hot plate (35-40°C) until the section was dry.

For a positive control paraffin embedded lung tissue sections containing foci known positive for viral antigen were run concurrently with LR White embedded tissue sections

- c) Paraffin sections were deparaffinized with xylene and rehydrated through graded alcohols. LR White sections were rehydrated with distilled water
- d) Antigen was unmasked by incubating in Tris-EDTA-Tween buffer for 10 minutes at room temperature.
- e) Blocked by flooding with normal goat sera in TBS/PBS<sup>b</sup> for 20 minutes at room temperature.
- f) Excess goat serum blotted off.
- g) Primary antibody (rabbit anti-RSV) applied and incubated overnight at 4°C.<sup>c</sup>

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<sup>a</sup> Polysciences Inc., Warrington, PA.

<sup>b</sup> One part 0.05M Tris buffered saline + 10 parts 0.01M phosphate buffered saline.

<sup>c</sup> All incubations were carried out in a humidified chamber.



- h) Rinsed by flushing 3 times with Tris-EDTA-Tween buffer.
- i) Secondary antibody (15 nm gold labeled goat anti-rabbit) applied diluted in Tris buffered saline + 1% BSA and incubated for 90 minutes at room temperature.
- j) Flushed 3 times with Tris-EDTA-Tween buffer.
- k) Silver enhancement performed (Intense M kit, Jansen Biotech N.V., Olen, Belgium), incubated until desired intensity was achieved.
- l) Washed in tap water.
- m) Counterstained for 1 minute with toluidine blue.

Results: There was no positive staining on paraffin sections in areas of known viral antigen presence.

Numerous LR White sections were lost during rinse prior counterstaining. Most likely resulting from the hydrophilic properties of LR White preventing adequate adherence of the sections to the slides.

The failure of attempts using immunogold silver enhancement to label known positive paraffin sections indicated that at the light microscopic level it was not as sensitive as the immunoperoxidase technique. It was decided to try the immunoperoxidase technique on LR White and epoxy embedded tissue sections.

IV. Respiratory syncytial virus antigen detection in LR White embedded lung tissue sections on acid cleaned slides using immunoperoxidase immunohistochemistry.

- a) Lung tissue from a sheep infected with BRSV was processed, infiltrated and embedded in LR White using thermal polymerization.
- b) 1  $\mu$ m LR White embedded tissue sections were floated onto acid cleaned slides using a drop of water or acetone. Sections were dried using at hot plate at a temperature of 60-65°C.
- c) Rehydrated with distilled water for 10 minutes at room temperature.
- d) Antigen was unmasked by incubating with Tris-EDTA-Tween buffer + 1% BSA for 15 minutes at room temperature.
- e) Blocked with normal goat sera for 15 minutes at 32°C.
- f) Excess goat sera blotted away.
- g) Primary antibody (rabbit anti-RSV) diluted in TBS was applied and incubated overnight at 4°C.
- h) Rinsed 3 times for 5 minutes each by submersion in Tris-EDTA-Tween buffer + 1% BSA at room temperature.
- i) Secondary antibody (goat anti-rabbit) diluted in TBS was applied and incubated for 45 minutes at 32°C.
- j) Rinsed by submersion 1 time for 5 minutes with Tris-EDTA-Tween buffer + 1% BSA then 2 times for 5 minutes each with TBS at room temperature.
- k) ABC reagent Vectastain ABC Kit, Vector Labs, Burlingame, CA) applied for 1 hour at 37°C.
- l) Rinsed once for 5 minutes with Tris-EDTA-Tween buffer then 2 times for 5 minutes each with TBS at room temperature.
- m) Incubated with 1% diaminobenzidine in 0.02% H<sub>2</sub>O<sub>2</sub> for 10 minutes at room temperature.
- n) Rinsed for 10 minutes with running tap water.

o) Counterstained with Mayer's hematoxylin for 3 minutes.

Negative controls consisted of substitution of normal rabbit sera for primary antibody

Results: Many sections floated off the slides regardless of water or acetone flotation of sections.

- V. Respiratory syncytial virus antigen detection in epoxy embedded lung sections on acid cleaned slides using immunoperoxidase.

Same protocol as used in IV.

Results: Many sections floated off the slides during the staining procedure

Excessive chromogen trapping within and under the treated tissues sections caused excessive background staining that prevented recognition of any specific staining.

- VI. Respiratory syncytial virus antigen detection in epoxy embedded section on poly-L-lysine coated slides.

- a) 1  $\mu\text{m}$  Tissue sections of 1  $\mu\text{m}$  thickness were floated onto poly-L-lysine coated slides.
- b) Remaining protocol identical to that of IV.

Results: Many sections floated off the slides during the staining procedure

Excessive chromogen trapping within and under the treated tissue sections caused excessive background staining that prevented recognition of any specific staining.

In an attempt to maximize viral antigen availability and labeling with immunogold, pre-embedding labeling techniques were attempted.

VII. Respiratory syncytial virus antigen detection using pre-embedding indirect colloidal gold immunocytochemical techniques on infected ovine turbinates grown on Millicell-HA membranes (Millipore Corp., Bedford, MA)

- a) Membranes were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer for 1 hour then stored in buffer.
- b) Rinsed by submersion in 0.1M cacodylate buffer.<sup>a</sup>
- c) Rinsed by submersion in TBS for 10 minutes.
- d) Antigen was unmasked by incubation with Tris-EDTA-Tween buffer for 10 minutes at room temperature.
- e) Rinsed 3 times for 5 minutes each with Tris-EDTA-Tween + 1% BSA at room temperature .
- f) Blocked with normal goat sera for 15 minutes at room temperature.
- g) Primary antibody (rabbit anti-RSV) diluted in TBS was applied and incubated overnight at 4°C.
- h) Rinsed 3 times for 5 minutes each with Tris-EDTA-Tween buffer + 1% BSA at room temperature.
- i) Gold labeled secondary antibody (15 nm gold labeled goat anti-rabbit) diluted in TBS was applied and incubated for 1 hour at 37°C.
- j) Rinsed 3 times for 5 minutes each with Tris-EDTA-Tween buffer + 1% BSA at room temperature .
- k) Rinsed 3 times for 5 minutes each with TBS at room temperature.
- l) Post-staining fixation with 3% glutaraldehyde in 0.1M cacodylate buffer and store.
- m) Processed for transmission electron microscopic examination.

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<sup>a</sup> All rinses were carried out by submersion on a rotary platform.

Negative controls consisted of non-infected ovine turbinates and substitution of normal rabbit sera for primary antibody.

Results: Staining was non-specific. Gold particles were present in low numbers randomly distributed over cellular and acellular portions of sections of infected tissues. Virions were present in treated tissues and were not labeled with gold particles.

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